



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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| <b>(21) International Application Number:</b> PCT/US92/08595<br><b>(22) International Filing Date:</b> 8 October 1992 (08.10.92)<br><b>(30) Priority data:</b><br>776,109 12 October 1991 (12.10.91) US<br>935,002 25 August 1992 (25.08.92) US<br><b>(60) Parent Applications or Grants</b><br>(63) Related by Continuation<br>US 07/776,109 (CIP)<br>Filed on 25 August 1992 (25.08.92)<br>US 07/935,002 (CIP)<br>Filed on 12 October 1991 (12.10.91)<br><b>(71) Applicant (for all designated States except US):</b> THE RE-<br>GENTS OF THE UNIVERSITY OF CALIFORNIA<br>[US/US]; 300 Lakeside Drive, 22nd Floor, Oakland, CA<br>94612-3550 (US).<br><b>(72) Inventors; and</b><br><b>(75) Inventors/Applicants (for US only) :</b> BUCHANAN, Bob, B.<br>[US/US]; 19 Tamalpias Road, Berkeley, CA 94708<br>(US). KOBREHEL, Karoly [FR/FR]; Laboratoire de<br>Technologie des Céréales (INRA), 2, place Viala, F-<br>34060 Montpellier Cédex 01 (FR). YEE, Boihon, C.  |           | [US/US]; 3215 Primrose Lane, Walnut Creek, CA 94598<br>(US). WONG, Joshua, H. [US/US]; 9 Viewmont Ter-<br>race, South San Francisco, CA 94080 (US). LOZANO,<br>Rosa [US/US]; 1155 Brighton Avenue, #16, Albany, CA<br>94706 (US). JIAO, Jin-an [CN/US]; 10555 West Flagler<br>Street, Miami, FL 33175 (US). SHIN, Sungho [KR/<br>KR]; Paek Kee Yoeup, Department of Horticulture,<br>Chungbuk National University, Cheongju 360-763 (KR).<br><b>(74) Agents:</b> SMITH, Karen, S. et al.; Flehr, Hohbach, Test,<br>Albritton & Herbert, 4 Embarcadero Center, Suite 3400,<br>San Francisco, CA 94111-4187 (US).<br><b>(81) Designated States:</b> AT, AU, BB, BG, BR, CA, CH, CS,<br>DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG,<br>MN, MW, NL, NO, PL, RO, RU, SD, SE, US, Euro-<br>pean patent (AT, BE, CH, DE, DK, ES, FR, GB, GR,<br>IE, IT, LU, MC, NL, SE), OAPI patent (BF, BJ, CF,<br>CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).<br><b>Published</b><br><i>With international search report.</i><br><i>Before the expiration of the time limit for amending the</i><br><i>claims and to be republished in the event of the receipt of</i><br><i>amendments.</i> |
| <b>(54) Title:</b> USE OF THIOL REDOX PROTEINS FOR REDUCING DISULFIDE BONDS<br><br><b>(57) Abstract</b><br><br>Methods of reducing cystine containing animal and plant proteins, and improving dough and baked goods' characteristics is provided which includes the steps of mixing dough with a thiol redox protein. The method of the present invention preferably uses reduced thioredoxin with wheat flour. Methods for reducing snake, bee and scorpion toxin proteins with a thiol redox (SH) agent and thereby inactivating the protein are also provided. Protease inhibitors are also reduced by the NADP/thioredoxin system. When two of these inhibitors, the Kunitz and Bowman-Birk trypsin inhibitors of soybean are reduced, they lose their ability to inhibit trypsin. Moreover, the reduced form of the inhibitors showed increased susceptibility to heat and proteolysis by either subtilisin or a protease preparation from germinating wheat seeds. A novel cystine containing protein that inhibits pullulanase was isolated. When it was reduced by thioredoxin its inhibitory activity was reduced. |           |   |

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## USE OF THIOL REDOX PROTEINS FOR REDUCING DISULFIDE BONDS

CROSS-REFERENCE

This application is a continuation-in-part of application Serial No. 07/776,109, filed October 12, 1991, and of application Serial No. 07/935,002, filed August 25, 1992  
5 which is a continuation-in-part application of Serial No. 07/776,109, filed October 12, 1991.

FIELD OF THE INVENTION

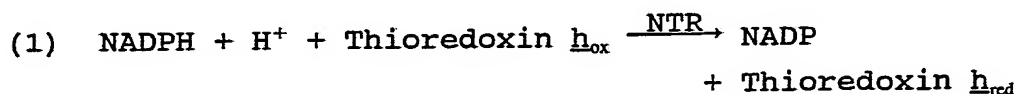
The present invention relates to the use of thiol redox proteins to reduce seed protein such as cereal proteins,  
10 enzyme inhibitor proteins, venom toxin proteins and the intramolecular disulfide bonds of certain other proteins. More particularly, the invention involves use of thioredoxin and glutaredoxin to reduce gliadins, glutenins, albumins and globulins to improve the  
15 characteristics of dough and baked goods and create new doughs and to reduce cystine containing proteins such as amylase and trypsin inhibitors so as to improve the quality of feed and cereal products. Additionally, the invention involves the isolation of a novel protein that  
20 inhibits pullulanase and the reduction of that novel protein by thiol redox proteins. The invention further

involves the reduction by thioredoxin of 2S albumin proteins characteristic of oil-storing seeds. Also, in particularly the invention involves the use of reduced thiol redox agents to inactivate snake neurotoxins and  
 5 certain insect and scorpion venom toxins *in vitro* and to treat the corresponding toxicities in individuals.

This invention was made with government support under Grant Contract Nos. DCB 8825980 and DMB 88-15980 awarded by the National Science Foundation. The United States  
 10 Government has certain rights in this invention.

#### BACKGROUND OF THE INVENTION

Chloroplasts contain a ferredoxin/thioredoxin system comprised of ferredoxin, ferredoxin-thioredoxin reductase and thioredoxins f and m that links light to the  
 15 regulation of enzymes of photosynthesis (Buchanan, B.B. (1991) "Regulation of CO<sub>2</sub> assimilation in oxygenic photosynthesis: The ferredoxin/thioredoxin system. Perspective on its discovery, present status and future development", Arch. Biochem. Biophys. 288:1-9; Scheibe,  
 20 R. (1991), "Redox-modulation of chloroplast enzymes. A common principle for individual control", Plant Physiol. 96:1-3). Several studies have shown that plants also contain a system, analogous to the one established for animals and most microorganisms, in which thioredoxin  
 25 (h-type) is reduced by NADPH and the enzyme, NADP-thioredoxin reductase (NTR) according to the following:

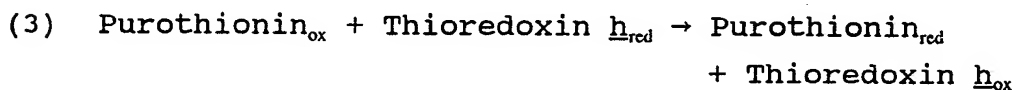
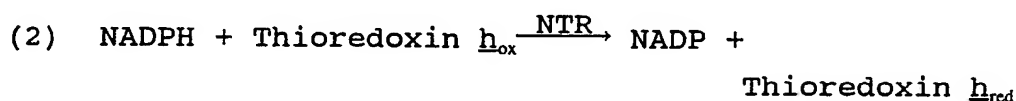


(Florencio F.J., *et al.* (1988), Arch. Biochem. Biophys. 266:496-507; Johnson, T.C., *et al.* (1987), Plant Physiol. 85:446-451; Suske, G., *et al.* (1979), Z. Naturforsch. C. 34:214-221). Current evidence suggests that the  
 30

NADP/thioredoxin system is widely distributed in plant tissues and is housed in the mitochondria, endoplasmic reticulum and cytosol (Bodenstein-Lang, J., et al. (1989), FEBS Lett. 258:22-26; Marcus, F., et al. (1991),  
 5 Arch. Biochem. Biophys. 287:195-198).

Thioredoxin h is also known to reductively activate cytosolic enzyme of carbohydrate metabolism, pyrophosphate fructose-6-P, 1-phosphotransferase or PFP (Kiss, F., et al. (1991), Arch. Biochem. Biophys.  
 10 287:337-340).

The seed is the only tissue for which the NADP/thioredoxin system has been ascribed physiological activity in plants. Also, thioredoxin h has been shown to reduce thionins in the laboratory (Johnson, T.C., et  
 15 al. (1987), Plant Physiol. 85:446-451). Thionins are soluble cereal seed proteins, rich in cystine. In the Johnson, et al. investigation, wheat purothionin was experimentally reduced by NADPH via NADP-thioredoxin reductase (NTR) and thioredoxin h according to Eqs. 2  
 20 and 3.



25 Cereal seeds such as wheat, rye, barley, corn, millet, sorghum and rice contain four major seed protein groups. These four groups are the albumins, globulins, gliadins and the glutenins or corresponding proteins. The thionins belong to the albumin group or faction.  
 30 Presently, wheat and rye are the only two cereals from which gluten or dough has been formed. Gluten is a tenacious elastic and rubbery protein complex that gives

cohesiveness to dough. Gluten is composed mostly of the gliadin and glutenin proteins. It is formed when rye or wheat dough is washed with water. It is the gluten that gives bread dough its elastic type quality. Flour  
5 from other major crop cereals barley, corn, sorghum, oat, millet and rice and also from the plant, soybean do not yield a gluten-like network under the conditions used for wheat and rye.

Glutenins and gliadins are cystine containing seed  
10 storage proteins and are insoluble. Storage proteins are proteins in the seed which are broken down during germination and used by the germinating seedling to grow and develop. Prolamines are the storage proteins in grains other than wheat that correspond to gliadins while  
15 the glutelins are the storage proteins in grains other than wheat that correspond to glutenins. The wheat storage proteins account for up to 80% of the total seed protein (Kasarda, D.D., et al. (1976), Adv. Cer. Sci. Tech. 1:158-236; and Osborne, T.B., et al. (1893), Amer. Chem. J. 15:392-471). Glutenins and gliadins are  
20 considered important in the formation of dough and therefore the quality of bread. It has been shown from *in vitro* experiments that the solubility of seed storage proteins is increased on reduction (Shewry, P.R., et al.  
25 (1985), Adv. Cer. Sci. Tech. 7:1-83). However, previously, reduction of glutenins and gliadins was thought to lower dough quality rather than to improve it (Dahle, L.K., et al. (1966), Cereal Chem. 43:682-688). This is probably because the non-specific reduction with  
30 chemical reducing agents caused the weakening of the dough.

The "Straight Dough" and the "Pre-Ferment" methods are two major conventional methods for the manufacture of dough and subsequent yeast raised bread products.

- For the Straight Dough method, all of the flour, water or other liquid, and other dough ingredients which may include, but are not limited to yeast, grains, salt, shortening, sugar, yeast nutrients, dough conditioners, and preservatives are blended to form a dough and are mixed to partial or full development. The resulting dough may be allowed to ferment for a period of time depending upon specific process or desired end-product characteristics.
- 10 The next step in the process is the mechanical or manual division of the dough into appropriate size pieces of sufficient weight to ensure achieving the targeted net weight after baking, cooling, and slicing. The dough pieces are often then rounded and allowed to rest
- 15 (Intermediate Proof) for varying lengths of time. This allows the dough to "relax" prior to sheeting and molding preparations. The time generally ranges from 5-15 minutes, but may vary considerably depending on specific processing requirements and formulations. The dough
- 20 pieces are then mechanically or manually formed into an appropriate shape are then usually given a final "proof" prior to baking. The dough pieces are then baked at various times, temperatures, and steam conditions in order to achieve the desired end product.
- 25 In the Pre-Ferment method, yeast is combined with other ingredients and allowed to ferment for varying lengths of time prior to final mixing of the bread or roll dough. Baker's terms for these systems include "Water Brew", "Liquid Ferment", "Liquid Sponge", and "Sponge/Dough".
- 30 A percentage of flour ranging from 0-100% is combined with the other ingredients which may include but are not limited to water, yeast, yeast nutrients and dough conditioners and allowed to ferment under controlled or ambient conditions for a period of time. Typical times
- 35 range from 1-5 hours. The ferment may then be used as

is, or chilled and stored in bulk tanks or troughs for later use. The remaining ingredients are added (flour, characterizing ingredients, additional additives, additional water, etc.) and the dough is mixed to partial  
5 or full development.

The dough is then allowed to ferment for varying time periods. Typically, as some fermentation has taken place prior to the addition of the remaining ingredients, the time required is minimal (*i.e.*, 10-20 min.), however,  
10 variations are seen depending upon equipment and product type. Following the second fermentation step, the dough is then treated as in the Straight Dough Method.

As used herein the term "dough mixture" describes a mixture that minimally comprises a flour or meal and a  
15 liquid, such as milk or water.

As used herein the term "dough" describes an elastic, pliable protein network mixture that minimally comprises a flour, or meal and a liquid, such as milk or water.

As used herein the term "dough ingredient" may include,  
20 but is not exclusive of, any of the following ingredients: flour, water or other liquid, grain, yeast, sponge, salt, shortening, sugar, yeast nutrients, dough conditioners and preservatives.

As used herein, the term "baked good" includes but is  
25 not exclusive of all bread types, including yeast-leavened and chemically-leavened and white and variety breads and rolls, english muffins, cakes and cookies, confectionery coatings, crackers, doughnuts and other sweet pastry goods, pie and pizza crusts, pretzels, pita  
30 and other flat breads, tortillas, pasta products, and refrigerated and frozen dough products.

While thioredoxin has been used to reduce albumins in flour, thiol redox proteins have not been used to reduce glutenins and gliadins nor other water insoluble storage proteins, nor to improve the quality of dough and baked goods. Thiol redox proteins have also not been used to improve the quality of gluten thereby enhancing its value nor to prepare dough from crop cereals such as barley, corn, sorghum, oat, millet and rice or from soybean flour.

Many cereal seeds also contain proteins that have been shown to act as inhibitors of enzymes from foreign sources. It has been suggested that these enzyme inhibitors may afford protection against certain deleterious organisms (Garcia-Olmedo, F., et al. (1987), Oxford Surveys of Plant Molecular and Cell Biology 4:275-335; Birk, Y. (1976), Meth. Enzymol. 45:695-739, and Laskowski, M., Jr., et al. (1980), Ann. Reo. Biochem. 49:593-626). Two such type enzyme inhibitors are amylase inhibitors and trypsin inhibitors. Furthermore, there is evidence that a barley protein inhibitor (not tested in this study) inhibits an  $\alpha$ -amylase from the same source (Weselake, R.J., et al. (1983), Plant Physiol. 72:809-812). Unfortunately, the inhibitor protein often causes undesirable effects in certain food products. The trypsin inhibitors in soybeans, notably the Kunitz trypsin inhibitor (KTI) and Bowman-Birk trypsin inhibitor (BBTI) proteins, must first be inactivated before any soybean product can be ingested by humans or domestic animals. It is known that these two inhibitor proteins become ineffective as trypsin inhibitors when reduced chemically by sodium borohydride (Birk, Y. (1985), Int. J. Peptide Protein Res. 25:113-131, and Birk, Y. (1976), Meth. Enzymol. 45:695-739). These inhibitors like other proteins that inhibit proteases contain intramolecular disulfides and are usually stable to inactivation by heat and proteolysis (Birk (1976), *supra.*; Garcia-Olmedo, et

al. (1987), *supra.*, and Ryan (1980). Currently, to minimize the adverse effects caused by the inhibitors these soybean trypsin inhibitors and other trypsin inhibitors in animal and human food products are being  
5 treated by exposing the food to high temperatures. The heat treatment, however, does not fully eliminate inhibitor activity. Further, this process is not only expensive but it also destroys many of the other proteins which have important nutritional value. For example,  
10 while 30 min at 120°C leads to complete inactivation of the BBTI of soy flour, about 20% of the original KTI activity remains (Friedman, et al., 1991). The prolonged or higher temperature treatments required for full inactivation of inhibitors results in destruction of  
15 amino acids such as cystine, arginine, and lysine (Chae, et al., 1984; Skrede and Krogdahl, 1985).

There are also several industrial processes which require  $\alpha$ -amylase activity. One example is the malting of barley which requires active  $\alpha$ -amylase. Inactivation of  
20 inhibitors such as the barley amylase/subtilisin (asi) inhibitor and its equivalent in other cereals by thiol redox protein reduction would enable  $\alpha$ -amylases to become fully active sooner than with present procedures, thereby shortening time for malting or similar processes.

25 Thiol redox proteins have also not previously been used to inactivate trypsin or amylase inhibitor proteins. The reduction of trypsin inhibitors such as the Kunitz and Bowman-Birk inhibitor proteins decreases their inhibitory effects (Birk, Y. (1985), Int. J. Peptide  
30 Protein Res. 25:113-131). A thiol redox protein linked reduction of the inhibitors in soybean products designed for consumption by humans and domestic animals would require no heat or lower heat than is presently required for protein denaturization, thereby cutting the costs  
35 of denaturation and improving the quality of the soy

protein. Also a physiological reductant, a so-called clean additive (*i.e.*, an additive free from ingredients viewed as "harmful chemicals") is highly desirable since the food industry is searching for alternatives to chemical additives. Further the ability to selectively reduce the major wheat and seed storage proteins which are important for flour quality (*e.g.*, the gliadins and the glutenins) in a controlled manner by a physiological reductant such as a thiol redox protein would be useful in the baking industry for improving the characteristics of the doughs from wheat and rye and for creating doughs from other grain flours such as cereal flours or from cassava or soybean flour.

The family of 2S albumin proteins characteristic of oil-storing seeds such as castor bean and Brazil nut (Kreis, *et al.* 1989; Youle and Huang, 1981) which are housed within protein bodies in the seed endosperm or cotyledons (Ashton, *et al.* 1976; Weber, *et al.* 1980), typically consist of dissimilar subunits connected by two intermolecular disulfide bonds -- one subunit of 7 to 9 kDa and the other of 3 to 4 kDa. The large subunit contains two intramolecular disulfide groups, the small subunit contains none. The intramolecular disulfides of the 2S large subunit show homology with those of the soybean Bowman-Birk inhibitor (Kreis, *et al.* 1989) but nothing is known of the ability of 2S proteins to undergo reduction under physiological conditions.

These 2S albumin proteins are rich in methionine. Recently transgenic soybeans which produce Brazil nut 2S protein have been generated. Reduction of the 2S protein in such soybeans could enhance the integration of the soy proteins into a dough network resulting in a soybread rich in methionine. In addition, these 2S proteins are often allergens. Reduction of the 2S

protein would result in the cessation of its allergic activity.

Pullulanase ("debranching enzyme") is an enzyme that breaks down the starch of the endosperm of cereal seeds by hydrolytically cleaving  $\alpha$ -1,6 bonds. Pullulanase is an enzyme fundamental to the brewing and baking industries. Pullulanase is required to break down starch in malting and in certain baking procedures carried out in the absence of added sugars or other carbohydrates. Obtaining adequate pullulanase activity is a problem especially in the malting industry. It has been known for some time that dithiothreitol (DTT, a chemical reductant for thioredoxin) activates pullulanase of cereal preparations (e.g., barley, oat and rice flours). A method for adequately activating or increasing the activity of pullulanase with a physiologically acceptable system, could lead to more rapid malting methods and, owing to increased sugar availability, to alcoholic beverages such as beers with enhanced alcoholic content.

Death and permanent injury resulting from snake bites are serious problems in many African, Asian and South American countries and also a major concern in several southern and western areas of the United States. Venoms from snakes are characterized by active protein components (generally several) that contain disulfide (S-S) bridges located in intramolecular (intrachain) cystines and in some cases in intermolecular (interchain) cystines. The position of the cystine within a given toxin group is highly conserved. The importance of intramolecular S-S groups to toxicity is evident from reports showing that reduction of these groups leads to a loss of toxicity in mice (Yang, C.C. (1967) Biochim. Biophys. Acta. 133:346-355; Howard, B.D., et al. (1977) Biochemistry 16:122-125). The neurotoxins of snake venom are proteins that alter the release of neurotransmitter

from motor nerve terminals and can be presynaptic or postsynaptic. Common symptoms observed in individuals suffering from snake venom neurotoxicity include swelling, edema and pain, fainting or dizziness, tingling  
5 or numbing of affected part, convulsions, muscle contractions, renal failure, in addition to long-term necrosis and general weakening of the individual, etc.

The presynaptic neurotoxins are classified into two groups. The first group, the  $\beta$ -neurotoxins, include three  
10 different classes of proteins, each having a phospholipase A<sub>2</sub> component that shows a high degree of conservation. The proteins responsible for the phospholipase A<sub>2</sub> activity have from 6 to 7 disulfide bridges. Members of the  $\beta$ -neurotoxin group are either  
15 single chain (e.g., caudotoxin, notexin and agkistrodotoxin) or multichain (e.g., crotoxin, ceruleotoxin and *Vipera* toxin).  $\beta$ -bungarotoxin, which is made up of two subunits, constitutes a third group. One of these subunits is homologous to the Kunitz-type  
20 proteinase inhibitor from mammalian pancreas. The multichain  $\beta$ -neurotoxins have their protein components linked ionically whereas the two subunits of  $\beta$ -bungarotoxin are linked covalently by an intermolecular disulfide. The B chain subunit of  $\beta$ -bungarotoxin, which  
25 is also homologous to the Kunitz-type proteinase inhibitor from mammalian pancreas, has 3 disulfide bonds.

The second presynaptic toxin group, the facilitatory neurotoxins, is devoid of enzymatic activity and has two subgroups. The first subgroup, the dendrotoxins, has  
30 a single polypeptide sequence of 57 to 60 amino acids that is homologous with Kunitz-type trypsin inhibitors from mammalian pancreas and blocks voltage sensitive potassium channels. The second subgroup, such as the fasciculins (e.g., fasciculin 1 and fasciculin 2) are

cholinesterase inhibitors and have not been otherwise extensively studied.

The postsynaptic neurotoxins are classified either as long or short neurotoxins. Each type contains S-S groups, but the peptide is unique and does not resemble either phospholipase A<sub>2</sub> or the Kunitz or Kunitz-type inhibitor protein. The short neurotoxins (e.g., erabutoxin a and erabutoxin b) are 60 to 62 amino acid residues long with 4 intramolecular disulfide bonds. The long neurotoxins (e.g.,  $\alpha$ -bungarotoxin and  $\alpha$ -cobratoxin) contain from 65 to 74 residues and 5 intramolecular disulfide bonds. Another type of toxins, the cytotoxins, acts postsynaptically but its mode of toxicity is ill defined. These cytotoxins show obscure pharmacological effects, e.g., hemolysis, cytolysis and muscle depolarization. They are less toxic than the neurotoxins. The cytotoxins usually contain 60 amino acids and have 4 intramolecular disulfide bonds. The snake venom neurotoxins all have multiple intramolecular disulfide bonds.

The current snake antitoxins used to treat poisonous snake bites following first aid treatment in individuals primarily involve intravenous injection of antivenom prepared in horses. Although it is not known how long after envenomation the antivenom can be administered and be effective, its use is recommended up to 24 hours. Antivenom treatment is generally accompanied by administration of intravenous fluids such as plasma, albumin, platelets or specific clotting factors. In addition, supporting medicines are often given, for example, antibiotics, antihistamines, antitetanus agents, analgesics and sedatives. In some cases, general treatment measures are taken to minimize shock, renal failure and respiratory failure. Other than administering calcium-EDTA in the vicinity of the bite and excising

the wound area, there are no known means of localized treatment that result in toxin neutralization and prevention of toxic uptake into the blood stream. Even these localized treatments are of questionable  
5 significance and are usually reserved for individuals sensitive to horse serum (Russell, F.E. (1983) Snake Venom Poisoning, Schollum International, Inc. Great Neck, NY).

The term "individual" as defined herein refers to an  
10 animal or a human.

Most of the antivenoms in current use are problematic in that they can produce harmful side effects in addition to allergic reactions in patients sensitive to horse serum (up to 5% of the patients). Nonallergic reactions  
15 include pyrogenic shock, and complement depletion (Chippaur, J.-P., et al. (1991) Reptile Venoms and Toxins, A.T. Tu, ed., Marcel Dekker, Inc., pp. 529-555).

It has been shown that thioredoxin, in the presence of NADPH and thioredoxin reductase reduces the bacterial  
20 neurotoxins tetanus and botulinum A *in vitro* (Schiavo, G., et al. (1990) Infection and Immunity 58:4136-4141; Kistner, A., et al. (1992) Naunyn-Schmiedeberg's Arch Pharmacol 345:227-234). Thioredoxin was effective in reducing the interchain disulfide link of tetanus toxin  
25 and such reduced tetanus toxin was no longer neurotoxic (Schiavo, et al., *supra.*). However, reduction of the interchain disulfide of botulinum A toxin by thioredoxin was reported to be much more sluggish (Kistner, et al., *supra.*). In contrast to the snake neurotoxin studied  
30 in the course of this invention, the tetanus research group (Schiavo, et al., *supra.*) found no evidence in the work done with the tetanus toxin that reduced thioredoxin reduced toxin intrachain disulfide bonds. There was also no evidence that thioredoxin reduced intrachain

disulfides in the work done with botulinum A. The tetanus and botulinum A toxins are significantly different proteins from the snake neurotoxins in that the latter (1) have a low molecular weight; (2) are rich  
5 in intramolecular disulfide bonds; (3) are resistant to trypsin and other animal proteases; (4) are active without enzymatic modification, e.g., proteolytic cleavage; (5) in many cases show homology to animal proteins, such as phospholipase A<sub>2</sub> and Kunitz-type  
10 proteases; (6) in most cases lack intermolecular disulfide bonds, and (7) are stable to agents such as heat and proteases.

Reductive inactivation of snake toxins *in vitro* by incubation with 1%  $\beta$ -mercaptoethanol for 6 hours and  
15 incubation with 8M urea plus 300mM  $\beta$ -mercaptoethanol has been reported in the literature (Howard, B.D., et al. (1977) Biochemistry 16:122-125; Yang, C.C. (1967) Biochim. Biophys. Acta. 133:346-355). These conditions, however, are far from physiological. As defined herein  
20 the term "inactivation" with respect to a toxin protein means that the toxin is no longer biologically active *in vitro*, in that the toxin is unable to link to a receptor. Also as used herein, "detoxification" is an extension of the term "inactivation" and means that the  
25 toxin has been neutralized in an individual as determined by animal toxicity tests.

Bee venom is a complex mixture with at least 40 individual components, that include major components as melittin and phospholipase A<sub>2</sub>, representing respectively  
30 50% and 12% of the total weight of the venom, and minor components such as small proteins and peptides, enzymes, amines, and amino acids.

Melittin is a polypeptide consisting of 26 amino acids with a molecular weight of 2840. It does not contain

a disulfide bridge. Owing to its high affinity for the lipid-water interphase, the protein permeates the phospholipid bilayer of the cell membranes, disturbing its organized structure. Melittin is not by itself a toxin but it alters the structure of membranes and thereby increases the hydrolytic activity of phospholipase A<sub>2</sub>, the other major component and the major allergen present in the venom.

Bee venom phospholipase A<sub>2</sub> is a single polypeptide chain of 128 amino acids, is cross-linked by four disulfide bridges, and contains carbohydrate. The main toxic effect of the bee venom is due to the strong hydrolytic activity of phospholipase A<sub>2</sub> achieved in association with melittin.

The other toxic proteins in bee venom have a low molecular weight and contain at least two disulfide bridges that seem to play an important structural role. Included are a protease inhibitor (63-65 amino acids), MCD or 401-peptide (22 amino acids) and apamin (18 amino acids).

Although there are thousands of species of bees, only the honey bee, *Apis mellifera*, is a significant cause of allergic reactions. The response ranges from local discomfort to systemic reactions such as shock, hypotension, dyspnea, loss of consciousness, wheezing and/or chest tightness that can result in death. The only treatment that is used in these cases is the injection of epinephrine.

The treatment of bee stings is important not only for individuals with allergic reactions. The "killer" or Africanized bee, a variety of honey bee is much more aggressive than European honey bees and represents a danger in both South and North America. While the

lethality of the venom from the Africanized and European bees appears to be the same (Schumacher, M. I., et al. (1989) Nature 337:413), the behaviour pattern of the hive is completely different. It was reported that  
5 Africanized bees respond to colony disturbance more quickly, in greater numbers and with more stinging (Collins, A. M., et al. (1982) Science 218:72-74). A mass attack by Africanized bees may produce thousands of stings on one individual and cause death. The  
10 "killer" bees appeared as a result of the interbreeding between the African bee (*Apis mellifera scutellata*) and the European bee (*Apis mellifera mellifera*). African bees were introduced in 1956 into Brazil with the aim of improving honey production being a more tropically  
15 adapted bee. Africanized bees have moved from South America to North America, and they have been reported in Texas and Florida.

In some areas of the world such as Mexico, Brazil, North Africa and the Middle East, scorpions present a life  
20 hazard to humans. However, only the scorpions of family Buthidae (genera, *Androctonus*, *Buthus*, *Centruroides*, *Leiurus* and *Tityus*) are toxic for humans. The chemical composition of the scorpion venom is not as complex as snake or bee venom. Scorpion venom contains  
25 mucopolysaccharides, small amounts of hyaluronidase and phospholipase, low molecular-weight molecules, protease inhibitors, histamine releasers and neurotoxins, such as serotonin. The neurotoxins affect voltage-sensitive ionic channels in the neuromuscular junction. The  
30 neurotoxins are basic polypeptides with three to four disulfide bridges and can be classified in two groups: peptides with from 61 to 70 amino acids, that block sodium channel, and peptides with from 36 to 39 amino acids, that block potassium channel. The reduction of  
35 disulfide bridges on the neurotoxins by nonphysiological reductants such as DTT or  $\beta$ -mercaptoethanol (Watt, D.

D., et al. (1972) Toxicon 10:173-181) lead to loss of their toxicity.

Symptoms of animals stung by poisonous scorpions include hyperexcitability, dyspnea, convulsions, paralysis and death. At present, antivenin is the only antidote for scorpion stings. The availability of the venom is a major problem in the production of antivenin. Unlike snake venom, scorpion venom is very difficult to collect, because the yield of venom per specimen is limited and in some cases the storage of dried venom leads to modification of its toxicity. An additional problem in the production of antivenins is that the neurotoxins are very poor antigens.

The reductive inactivation of snake, bee and scorpion toxins under physiological conditions has never been reported nor has it been suggested that the thiol redox agents, such as reduced lipoic acid, DTT, or reduced thioredoxin could act as an antidote to these venoms in an individual.

## 20 SUMMARY OF THE INVENTION

It is an object herein to provide a method for reducing a non thionin cystine containing protein.

It is a second object herein to provide methods utilizing a thiol redox protein alone or in combination with a reductant or reduction system to reduce glutenins or gliadins present in flour or seeds.

It is also an object herein to provide methods using a thiol redox protein alone or in combination with a reductant or reduction system to improve dough strength and baked goods characteristics such as better crumb

quality, softness of the baked good and higher loaf volume.

It is a further object herein to provide formulations containing a thiol redox protein useful in practicing  
5 such methods.

Still a further object herein is to provide a method for producing a dough from rice, corn, soybean, barley, oat, cassava, sorghum or millet flour.

Yet, another object is to provide a method for producing  
10 an improved gluten or for producing a gluten-like product from cereal grains other than wheat and rye.

It is further an object herein to provide a method of reducing an enzyme inhibitor protein having disulfide bonds.

15 Still another object herein is to provide yeast cells genetically engineered to express or overexpress thioredoxin.

Still yet another object herein is to provide yeast cells genetically engineered to express or overexpress NADP-  
20 thioredoxin reductase.

Still yet a further object herein is to provide a method for improving the quality of dough or a baked good using such genetically engineered yeast cells.

Yet still another object herein is to provide a method  
25 of reducing the intramolecular disulfide bonds of a non-thionin, non chloroplast protein containing more than one intramolecular cystine comprising adding a thiol redox protein to a liquid or substance containing the cystines containing protein, reducing the thiol redox

protein and reducing the cystines containing protein by means of the thiol redox protein.

Another object herein is to provide an isolated pullulanase inhibitor protein having disulfide bonds and  
5 a molecular weight of between 8 to 15 kDa.

Still another object herein is to provide a method of increasing the activity of pullulanase derived from barley or wheat endosperm comprising adding thioredoxin to a liquid or substance containing the pullulanase and  
10 reducing the thioredoxin thereby increasing the pullulanase activity.

Still another object herein is to provide a method of reducing an animal venom toxic protein having one or more intramolecular cystines comprising contacting the cystine  
15 containing protein with an amount of a thiol redox (SH) agent effective for reducing the protein, and maintaining the contact for a time sufficient to reduce one or more disulfide bridges of the one or more intramolecular cystines thereby reducing the neurotoxin protein. The  
20 thiol redox (SH) agent may be a reduced thioredoxin, reduced lipoic acid in the presence of a thioredoxin, DTT or DTT in the presence of a thioredoxin and the snake neurotoxin protein may be a presynaptic or postsynaptic neurotoxin.

25 Still a further object of the invention is to provide a composition comprising a snake neurotoxin protein and a thiol redox (SH) agent.

Still yet another object of the invention is to provide a method of reducing an animal venom toxic protein having  
30 one or more intramolecular cystines comprising contacting the protein with amounts of NADP-thioredoxin reductase, NADPH or an NADPH generator system and a thioredoxin

effective for reducing the protein, and maintaining the contact for a time sufficient to reduce one or more disulfide bridges of the one or more intramolecular cystines thereby reducing the protein.

- 5 Yet another object herein is to provide a method of inactivating, *in vitro*, a snake neurotoxin having one or more intramolecular cystines comprising adding a thiol redox (SH) agent to a liquid containing the toxin wherein the amount of the agent is effective for reducing the  
10 toxin.

- Yet a further object herein is to provide a method of treating venom toxicity in an individual comprising administering, to an individual suffering from venom toxicity, amounts of a thiol redox (SH) agent effective  
15 for reducing or alleviating the venom toxicity.

In accordance with the objects of the invention, methods are provided for improving dough characteristics comprising the steps of mixing a thiol redox protein with dough ingredients to form a dough and baking said dough.

- 20 Also, in accordance with the objects of the invention, a method is provided for inactivating an enzyme inhibitor protein in a grain food product comprising the steps of mixing a thiol redox protein with the seed product, reducing the thiol redox protein by a reductant or  
25 reduction system and reducing the enzyme inhibitor by the reduced thiol redox protein, the reduction of the enzyme inhibitor inactivating the enzyme inhibitor.

- The thiol redox proteins in use herein can include thioredoxin and glutaredoxin. The thioredoxin includes  
30 but is not exclusive of *E. coli* thioredoxin, thioredoxin h, f and m and animal thioredoxins. A reductant of thioredoxin used herein can include lipoic acid or a

reduction system such as NADPH in combination with NADP  
thioredoxin reductase (NTR). The reductant of  
glutaredoxin can include reduced glutathione in  
conjunction with the reduction system NADPH and  
5 glutathione reductase. NADPH can be replaced with an  
NADPH generator or generator composition such as one  
consisting of glucose 6-phosphate, NADP and glucose 6-  
phosphate dehydrogenase from a source such as yeast.  
The NADPH generator is added together with thioredoxin  
10 and NADP-thioredoxin reductase at the start of the dough  
making process.

It should be noted that the invention can also be  
practiced with cysteine containing proteins. The  
cysteines can first be oxidized and then reduced via  
15 thiol redox protein.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 depicts a graph showing the effect of  $\alpha$ -amylase  
protein inhibitors on activation of NADP-Malate  
Dehydrogenase in the presence of DTT-reduced  
20 Thioredoxin h.

Fig. 2 is a graph showing the effect of  $\alpha$ -amylase  
Inhibitor Concentration on NADP-Malate Dehydrogenase  
Activation by  $\alpha$ -amylase Inhibitors.

Fig. 3 is a graph showing the effect of Thioredoxin h  
25 Concentration on Activation of NADP-Malate dehydrogenase  
by DSG-1 or -2  $\alpha$ -Amylase Inhibitors.

Fig. 4 is a graph showing the effect of  $\alpha$ -Amylase  
Inhibitors on DTNB Reduction by the *E. coli*  
NADP/Thioredoxin System.

Fig. 5 is a graph showing the effect of purothionin  $\alpha$  and CM-1  $\alpha$ -Amylase Inhibitor from Bread Wheat on DTNB Reduction by the *E. coli* NADP/Thioredoxin System.

5 Fig. 6 is a photograph taken of an SDS polyacrylamide electrophoretic gel placed over a long UV wavelength light box showing the Thioredoxin-Linked Reduction of Soluble Sulfur Rich Seed Proteins: Durum Wheat  $\alpha$ -Amylase Inhibitor (DSG-1) and Bowman-Birk Soybean Trypsin Inhibitor (BBTI).

10 Fig. 7 is a photograph taken of an SDS polyacrylamide electrophoretic gel placed over a long UV wavelength light box showing the Thioredoxin-Linked Reduction of Seed Proteins.

15 Fig. 8 is a photograph taken of an SDS-polyacrylamide electrophoretic gel placed over a fluorescent light box showing Thioredoxin-linked reduction of gliadins determined by an SDS-PAGE/mBBR procedure.

20 Fig. 9 is a photograph taken of an acidic-polyacrylamide electrophoretic gel placed over a fluorescent light box showing Thioredoxin-linked reduction of the different types of gliadins determined by an acid PAGE/mBBR procedure.

25 Fig. 10 is a photograph taken of an SDS-polyacrylamide electrophoretic gel placed over a fluorescent light box showing Thioredoxin-linked reduction of acid soluble glutenins determined by an SDS-PAGE/mBBR procedure.

Fig. 11 is a graph showing the relative reduction of seed protein fractions during germination.

Fig. 12 is a bar graph showing reduction of principal thioredoxin-linked gliadins and glutenins during germination (compared with *in vivo* reduction).

Fig. 13 is a diagrammatic representation of the proposed  
5 role of thioredoxin in forming a protein network for bread and pasta.

Fig. 14 shows farinograms of treated and untreated medium quality flour; Fig. 14(a) is the farinogram of the medium quality flour; Fig. 14(b) is of the same flour following  
10 treatment with reduced glutathione, and Fig. 14(c) is of the medium quality flour after treatment with the NADP/thioredoxin system.

Fig. 15 shows farinograms of treated and untreated poor quality flour; Fig. 15(a) is a farinogram of the poor  
15 quality flour control; Fig. 15(b) is of the same flour after treatment with reduced glutathione, and Fig. 15(c) is of the poor quality flour after treatment with DTT, and Fig. 15(d) is of the poor quality flour after treatment with the NADP/thioredoxin system.

20 Fig. 16 shows farinograms of treated and untreated Apollo flour; Fig. 16(a) represents the untreated flour, and Fig. 16(b) represents the same flour treated with the NTS.

Fig. 17 shows farinograms of treated and untreated Apollo  
25 flour; Fig. 17(a) is a farinogram of the Apollo control; Fig. 17(b) is of the same flour after treatment with an NADPH generating system; Fig. 17(c) is of the Apollo flour after treatment with the same generating system plus NTR and thioredoxin.

Fig. 18 is a photograph showing a top view of a comparison between an Arbon loaf of bread made from thioredoxin-treated dough and an untreated control loaf.

5 Fig. 19 is a photograph showing a side elevational and partial top view of a comparison between a loaf made from a thioredoxin-treated Arbon flour dough and an untreated control loaf.

10 Fig. 20 is a photograph showing a side elevational view of a comparison between a loaf made from a thioredoxin-treated Arbon flour dough and an untreated control loaf.

Fig. 21 is a photograph showing a top view of a comparison between a loaf made from a thioredoxin-treated Arbon flour dough and an untreated control loaf.

15 Fig. 22 is a photograph showing a side elevational and partial top view of a comparison between a loaf made from a thioredoxin-treated Arbon flour dough and an untreated control loaf.

20 Fig. 23 shows photographs comparing breads baked from thioredoxin treated and untreated doughs; Fig. 23(a) shows a comparison of loaves of bread made from treated and untreated arbon flour, and Fig. 23(b) shows a comparison among baked goods that were prepared from thioredoxin-treated and untreated corn, rice and sorghum flour.

25 Fig. 24 is a photograph showing a top and partial side view of a comparison between a loaf baked from a triticale flour dough treated with thioredoxin and a control loaf made from untreated triticale flour dough.

Fig. 25 is a photograph showing comparisons among baked goods that were prepared from thioredoxin-treated and untreated corn, rice and sorghum flour.

Fig. 26 represents photographs of an SDS polyacrylamide electrophoretic gel showing the extent of thioredoxin-linked reduction of myristate-extracted proteins from oat flour.

Fig. 27 represents photographs of an SDS polyacrylamide electrophoretic gel showing the extent of thioredoxin-linked reduction of myristate-extracted proteins from triticales flour.

Fig. 28 represents photographs of an SDS polyacrylamide electrophoretic gel showing the extent of thioredoxin-linked reduction of myristate-extracted proteins from rye flour.

Fig. 29 represents photographs of an SDS polyacrylamide electrophoretic gel showing the extent of thioredoxin-linked reduction of myristate-extracted proteins from barley flour.

Fig. 30 represents photographs of an SDS polyacrylamide electrophoretic gel showing the extent of thioredoxin-linked reduction of buffer, ethanol and myristate-extracted proteins from teff flour; Fig. 30(a) shows fluorescence and Fig. 30(b) shows the protein staining.

Fig. 31 is a photograph of an SDS polyacrylamide electrophoretic gel showing the effect of NTS vs. glutathione reductase on the reduction status of myristate-extracted proteins from corn, sorghum and rice.

Fig. 32 is a photograph of an SDS polyacrylamide electrophoretic gel showing the *in vivo* reduction status and thioredoxin-linked *in vitro* reduction of myristate-extracted proteins from corn, sorghum and rice.

- 5 Fig. 33 represents photographs of an SDS polyacrylamide electrophoretic gel showing the relative reduction of wheat glutenins by a yeast NADP/thioredoxin system.

- Fig. 34 represents photographs of an SDS polyacrylamide electrophoretic gel showing the relative reduction of  
10 wheat gliadins by a yeast NADP/thioredoxin system.

Fig. 35 represents photographs of an SDS polyacrylamide electrophoretic gel showing the extent of thioredoxin-linked reduction of ethanol-extracted proteins from triticales flour.

- 15 Fig. 36 represents photographs of an SDS polyacrylamide electrophoretic gel showing the extent of thioredoxin-linked reduction of ethanol-extracted proteins from rye flour.

- Fig. 37 represents photographs of an SDS polyacrylamide  
20 electrophoretic gel showing the extent of thioredoxin-linked reduction of ethanol-extracted proteins from oat flour.

- Fig. 38 represents photographs of an SDS polyacrylamide electrophoretic gel showing the extent of thioredoxin-  
25 linked reduction of ethanol-extracted proteins from barley flour.

- Fig. 39 represents photographs of an SDS polyacrylamide electrophoretic gels showing the extent of reduction of castor seed matrix and crystalloid proteins by various  
30 reductants.

Fig. 40 is a photograph of an SDS polyacrylamide electrophoretic gel showing the reduction specificity of 2S proteins.

Fig. 41 is a graph showing the separation of pullulanase  
5 inhibitor from pullulanase of barley malt by DE52 chromatography.

Fig. 42 is a graph showing the purification of pullulanase inhibitor of barley malt by CM32 chromatography at pH 4.6.

10 Fig. 43 is a graph showing the purification of pullulanase inhibitor of barley malt by CM32 chromatography at pH 4.0.

Fig. 44 is a graph showing the purification of pullulanase inhibitor of barley malt by Sephadex G-75  
15 chromatography.

Fig. 45 represents photographs of SDS polyacrylamide electrophoretic gels showing the extent of reduction of bee venom proteins by various reductants.

Fig. 46 represents photographs of SDS polyacrylamide  
20 electrophoretic gels showing the extent of reduction of scorpion venom proteins by various reductants.

Fig. 47 represents photographs of SDS polyacrylamide electrophoretic gels showing the extent of reduction of snake venom proteins by various reductants.

25 Fig. 48 represents photographs of an SDS polyacrylamide electrophoretic gel showing the extent of reduction of bee, scorpion and snake venom proteins with the NTS in the presence and absence of protease inhibitors.

Fig. 49 is a photograph of an SDS polyacrylamide electrophoretic gel showing the extent of reduction of erabutoxin b samples treated with different reductants.

Fig. 50 is a graph showing the activation of chloroplast NADP-malate dehydrogenase by erabutoxin b reduced with different thioredoxins compared to the activation of the dehydrogenase by a control lacking toxin.

Fig. 51 is a graph showing the effect of thioredoxin-linked reduction of  $\beta$ -bungarotoxin on  $\beta$ -bungarotoxin phospholipase A<sub>2</sub> activity.

Fig. 52 is a photograph of an SDS polyacrylamide electrophoretic gel showing the extent of reduction of  $\beta$ -bungarotoxin and  $\alpha$ -bungarotoxin samples with cellular reductants.

#### 15 DETAILED DESCRIPTION OF THE INVENTION

In accordance with this detailed description, the following definitions and abbreviations apply:

|                |  |
|----------------|--|
| CM             | - certain bread wheat $\alpha$ -amylase inhibitors   |
| DSG            | - certain $\alpha$ -amylase inhibitors isolated from |
| 20 durum wheat |  |
| DTNB           | - 2'5'-dithiobis (2-nitrobenzoic acid)               |
| NTR            | - NADP-thioredoxin reductase                         |
| mBBr           | - monobromobimane                                    |
| NADP-MDH       | - NADP-malate dehydrogenase                          |
| 25 FBPase      | - fructose-1,6-bisphosphatase                        |
| SDS            | - sodium dodecyl sulfate                             |
| DTT            | - dithiothreitol                                     |
| Cereal         | - millet, wheat, oat, barley, rice, sorghum,         |
|                | or rye   |
| 30 BBTI        | - Bowman-Birk soybean trypsin inhibitor              |
| KTI            | - Kunitz soybean trypsin inhibitor                   |

PAGE - polyacrylamide gel electrophoresis  
TCA - trichloroacetic acid

### Enzyme Inhibitor Protein Experiments

#### Starting Materials

#### 5 Materials

Seeds of bread wheat Triticum aestivum L, cv. Talent) and durum wheat (Triticum durum. Desf., cv. Mondur) were obtained from laboratory stocks.

#### Reagents

- 10 Chemicals and fine chemicals for enzyme assays and sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis were purchased from Sigma Chemical Co. and BioRad Laboratories, respectively. Monobromobimane (mBBr, tradename Thiolite) was purchased from Calbiochem. Other  
15 chemicals were obtained from commercial sources and were of the highest quality available.

#### Enzymes

- Thiorodoxin and NTR from E. coli were purchased from American Diagnostics, Inc. and were also isolated from  
20 cells transformed to overexpress each protein. The thiorodoxin strain containing the recombinant plasmid, pFP1, was kindly provided by Dr. J.-P. Jacquot (de la Motte-Guery, F. et al. (1991) Eur. J. Biochem. 196:287-294). The NTR strain containing the recombinant plasmid,  
25 pPMR21, was kindly provided by Drs. Marjorie Russel and Peter Model (Russel, M. et al. (1988) J. Biol. Chem. 263:9015-9019). The Isolation procedure used for these proteins was as described in those studies with the following changes: cells were broken in a Ribi cell  
30 fractionator at 25,000 psi and NTR was purified as described by Florencio et al. (Florencio, F. J. et al. (1988) Arch. Biochem. Biophys. 266:496-507) without the

red agarose step. Thioredoxin and NTR from *Saccharomyces cerevisiae* (baker's yeast type 1) were isolated by the procedure developed by Florencio, et al. for spinach leaves with the following changes: suspended cells [1  
5 part cells:5 parts buffer (w/v)], were broken in a Ribi cell fractionator at 40,000 psi with three passes.

Thioredoxin h and NTR were isolated from wheat germ by the procedure developed for spinach leaves (Florencio, F.J., et al. (1988), Arch. Biochem. Biophys. 266:496-  
10 507). NADP-malate dehydrogenase (NADP-MDH) and fructose-1,6-bisphosphatase (FBPase) were purified from leaves of corn (Jacquot, J.-P., et al. (1981), Plant Physiol. 68:300-304) and spinach (Crawford, N.A., et al. (1989), Arch. Biochem. Biophys. 271:223-239) respectively. *E.*  
15 *coli* glutaredoxin and calf thymus thioredoxin were obtained from Professor A. Holmgren.

#### $\alpha$ -Amylase and trypsin inhibitors

CM-1 protein was isolated from the albumin-globulin fraction of bread wheat flour as described previously  
20 (Kobrehel, K., et al. (1991), Cereal Chem. 68:1-6). A published procedure was also used for the isolation of DSG proteins (DSG-1 and DSG-2) from the glutenin fraction of durum wheat (Kobrehel, K. et al. (1989), J. Sci. Food Agric. 48:441-452). The CM-1, DSG-1 and DSG-2 proteins  
25 were homogeneous in SDS-polyacrylamide gel electrophoresis. Trypsin inhibitors were purchased from Sigma Chemical Co., except for the one from corn kernel which was from Fluca. In all cases, the commercial preparations showed a single protein component which  
30 migrated as expected in SDS-PAGE (Coomassie Blue stain), but in certain preparations, the band was not sharp.

#### Other proteins

Purothionin  $\alpha$  from bread wheat and purothionins  $\alpha$ -1 and  $\beta$  from durum wheat were kind gifts from Drs. D.D. Kasarda

and B.L. Jones, respectively. The purothionin  $\alpha$  sample contained two members of the purothionin family when examined with SDS-polyacrylamide gel electrophoresis. The purothionin  $\alpha$ -1 and  $\beta$  samples were both homogeneous  
5 in SDS-polyacrylamide gel electrophoresis.

#### Routine Method Steps

##### Enzyme activation assays

The NADP-MDH, FBPase, NTR and Thioredoxin h assay methods were according to Florencio, F.J., et al. (1988), Arch. Biochem. Biophys. 266:496-507 with slight modifications  
10 as indicated. For enzyme activation assays, the preincubation time was 20 min. unless specified otherwise.

##### mBBR Fluorescent labeling and SDS-polyacrylamide gel electrophoresis analyses

Direct reduction of the proteins under study was determined by a modification of the method of Crawford, et al. (Crawford, N.A., et al. (1989), Arch. Biochem. Biophys. 271:223-239). The reaction was carried out in  
20 100 mM potassium phosphate buffer, pH 7.1, containing 10 mM EDTA and 16% glycerol in a final volume of 0.1 ml. As indicated, 0.7  $\mu$ g (0.1  $\mu$ M) NTR and 1  $\mu$ g (0.8  $\mu$ M) thioredoxin (both routinely from *E. coli*) were added to 70  $\mu$ l of the buffer solution containing 1 mM NADPH and  
25 10  $\mu$ g (2 to 17  $\mu$ M) of target protein. When thioredoxin was reduced by dithiothreitol (DTT, 0.5 mM), NADPH and NTR were omitted. Assays with reduced glutathione were performed similarly, but at a final concentration of 1 mM. After incubation for 20 min, 100 nmoles of mBBR were  
30 added and the reaction was continued for another 15 min. To stop the reaction and derivatize excess mBBR, 10  $\mu$ l of 10% SDS and 10  $\mu$ l of 100 mM  $\beta$ -mercaptoethanol were added and the samples were then applied to the gels. In the case of reduction by glutaredoxin, the thioredoxin  
35 and NTR were replaced by 1  $\mu$ g (0.8  $\mu$ M) *E. coli*

glutaredoxin, 1.4  $\mu$ g (0.14  $\mu$ M) glutathione reductase purified from spinach leaves (Florencio, F.J., et al. (1988), Arch. Biochem. Biophys. 266:496-507) and 1.5 mM NADPH was used.

5 Gels (17.5% w/v, 1.5 mm thickness) were prepared according to Laemmli (Laemmli, U.K. (1970), Nature 227:680-685) and developed for 16 hr. at constant current (9 mA). Following electrophoresis, gels were placed in a solution of 40% methanol and 10% acetic acid, and  
10 soaked for 4 to 6 hours with several changes of the solution. Gels were then examined for fluorescent bands with near ultraviolet light and photographed (exposure time 25 sec) according to Crawford, et al. (Crawford, N.A., et al. (1989), Arch. Biochem. Biophys. 271:223-  
15 239). Finally, gels were stained with Coomassie Blue and destained as before (Crawford, N.A., et al. (1989), Arch. Biochem. Biophys. 271:223-239).

#### Quantification of labeled proteins

To obtain a quantitative indication of the extent of  
20 reduction of test proteins by the NADP/thioredoxin system, the intensities of their fluorescent bands seen in SDS-polyacrylamide gel electrophoresis were evaluated, using a modification of the procedure of Crawford, et al. (Crawford, N.A., et al. (1989), Arch. Biochem.  
25 Biophys. 271:223-239). The photographic negatives were scanned using a Pharmacia Ultrascan laser densitometer, and the area underneath the peaks was quantitated by comparison to a standard curve determined for each protein. For the latter determination, each protein (at  
30 concentrations ranging from 1 to 5  $\mu$ g) was reduced by heating for 3 min. at 100°C in the presence of 0.5 mM DTT. Labeling with mBBR was then carried out as described above except that the standards were heated for 2 min. at 100°C after the reaction was stopped with  
35 SDS and excess mBBR derivatized with  $\beta$ -mercaptoethanol.

Because the intensity of the fluorescent bands was proportional to the amounts of added protein, it was assumed that reduction was complete under the conditions used.

EXAMPLE 1Thioredoxin-linked Reduction of  $\alpha$ -Amylase InhibitorsEnzyme Activation Assays

The capability to replace a specific thioredoxin in the  
5 activation of chloroplast enzymes is one test for the  
ability of thiol groups of a given protein to undergo  
reversible redox change. Even though not physiological  
in the case of extraplastidic proteins, this test has  
proved useful in several studies. A case in point is  
10 purothionin which, when reduced by thioredoxin h  
activates chloroplast FBPase (Wada, K. et al. (1981),  
FEBS Lett., 124:237-240, and Johnson, T.C., et al.  
(1987), Plant Physiol., 85:446-451). The FBPase, whose  
physiological activator is thioredoxin f, is unaffected  
15 by thioredoxin h. In this Example, the ability of  
cystine-rich proteins to activate FBPase as well as  
NADP-MDH was tested as set forth above. The  $\alpha$ -amylase  
inhibitors from durum wheat (DSG-1 and DSG-2) were found  
to be effective in enzyme activation; however, they  
20 differed from purothionin in showing a specificity for  
NADP-MDH rather than FBPase (Table I). The  $\alpha$ -amylase  
inhibitors were active only in the presence of reduced  
thioredoxin h, which itself did not significantly  
activate NADP-MDH under these conditions (Fig. 1). As  
25 shown in Fig. 1, DSG-1 and DSG-2 activated NADP-malate  
dehydrogenase in the presence of DTT-reduced thioredoxin  
h according to the reaction sequence  
(DTT  $\rightarrow$  Thioredoxin  $\rightarrow$  DSG  $\rightarrow$  NADP-MDH).

Fig. 1 represents results obtained with either the DSG-1  
30 or DSG-2 inhibitors from durum wheat;  $\beta$ -MET refers to  
 $\beta$ -mercaptoethanol. The complete system for activation  
contained in 200  $\mu$ l of 100 mM Tris-HCl buffer, pH 7.9;  
10 mM DTT; 0.7  $\mu$ g corn leaf NADP-MDH; 0.25  $\mu$ g wheat  
thioredoxin h and 10  $\mu$ g of DSG-1 or DSG-2. As indicated,  
35 20 mM  $\beta$ -mercaptoethanol ( $\beta$ -MET) replaced DTT. Following

preincubation, NADP-MDH was assayed spectrophotometrically.

In the enzyme activation assays, thioredoxin h was reduced by DTT; as expected, monothiols such as  $\beta$ -mercaptoethanol ( $\beta$ -MET), which do not reduce thioredoxin at a significant rate under these conditions (Jacquot, J.-P., et al. (1981), Plant Physiol. 68:300-304; Nishizawa, A.N., et al. (1982), "Methods in Chloroplast Molecular Biology", (M. Edelman, R.B. Hallick and N.-H. Chua, eds.) pp. 707-714, Elsevier Biomedical Press, New York, and Crawford, N.A., et al. (1989), Arch. Biochem. Biophys. 271:223-239), did not replace DTT.

NADP-MDH activity was proportional to the level of added DSG-1 and DSG-2 at a constant thioredoxin h concentration (see Fig. 2). Fig. 2 shows the effect of  $\alpha$ -amylase inhibitor concentration on NADP-malate dehydrogenase activation by DSG-1 and DSG-2 according to the same DTT formula set forth above. Except for varying the DSG-1 or DSG-2 concentrations, conditions were identical to those previously described and shown in Fig. 1. When tested at a fixed DSG concentration, NADP-MDH showed enhanced activity with increasing thioredoxin h (as shown in Fig. 3). Except for varying the thioredoxin h concentration, conditions were as described above for Fig. 1.

CM-1--the bread wheat protein that is similar to DSG proteins but has a lower molecular weight - also activated NADP-MDH and not FBPase when 20 $\mu$ g of CM-1 were used as shown in Table I. The results indicate that thioredoxin h reduces a variety of  $\alpha$ -amylase inhibitors, which, in turn, activate NADP-MDH in accordance with equations 4-6. These proteins were ineffective in

enzyme activation when DTT was added in the absence of thioredoxin.

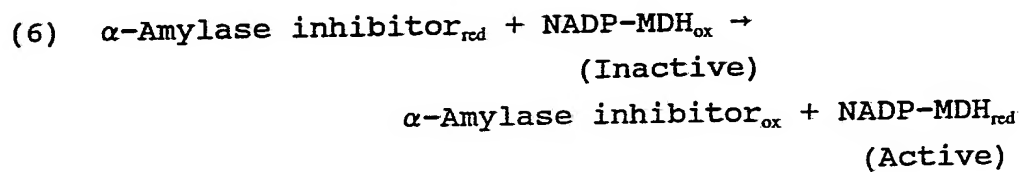
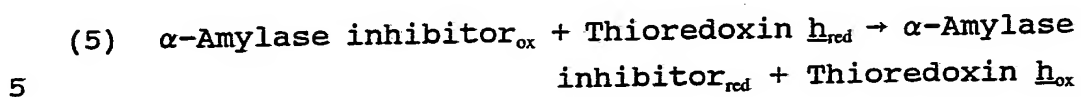
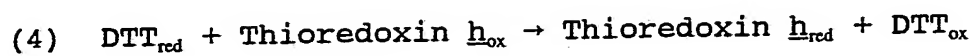


TABLE I

Effectiveness of Thioredoxin-Reduced  
Trypsin Inhibitors, Thionins, and  $\alpha$ -Amylase  
Inhibitors in Activating Chloroplast NADP-Malate  
Dehydrogenase and Fructose Bisphosphatase  
(DTT→Thioredoxin→Indicated Protein→Target Enzyme)

Activation of NADPH-MDH was carried out as in Fig. 1 except that the quantity of DSG or the other proteins tested was 20  $\mu$ g. FBPase activation was tested using the standard DTT assay with 1  $\mu$ g of *E. coli* thioredoxin and 20  $\mu$ g of the indicated proteins. The above values are corrected for the limited activation seen with *E. coli* thioredoxin under these conditions (see Fig. 1).

| 15 | <u>Protein</u>                                | <u>M, kDa</u> | <u>No. of<br/>S-S Groups</u> | <u>*ACTIVITY, nkat/mg</u><br><u>NADP-MDH</u> | <u>FBPase</u> |
|----|---|---------------|------------------------------|--|---------------|
|    | <u><math>\alpha</math>-Amylase Inhibitors</u> |               |                              |  |               |
|    | **DSG-2                                       | 17            | 5                            | 2  | 0             |
|    | **DSG-1                                       | 14            | 5                            | 2  | 0             |
| 20 | ‡CM-1   | 12            | 5                            | 12   | 0             |
|    | <u>Trypsin Inhibitors</u>                     |               |                              |  |               |
|    | <u>Cystine-rich (plant)</u>                   |               |                              |  |               |
|    | Corn kernel                                   | 12            | 5                            | 5  | 0             |
|    | Soybean Bowman-Birk                           | 8             | 7                            | 3  | 0             |
| 25 | <u>Other types</u>                            |               |                              |  |               |
|    | Ovomucoid                                     | 28            | 9                            | 2  | 0             |
|    | Soybean Kunitz                                | 20            | 2                            | 2  | 0             |
|    | Ovoinhibitor                                  | 49            | 14                           | 1  | 0             |
|    | Bovine lung (Aprotinin)                       | 7             | 3                            | Trace  | 2             |
| 30 | <u>Thionins</u>                               |               |                              |  |               |
|    | **Purothionin- $\alpha_1$                     | 6             | 4                            | 1  | 39            |
|    | **Purothionin- $\beta$                        | 6             | 4                            | Trace  | 5             |
|    | ‡Purothionin- $\alpha$                        | 6             | 4                            | 0  | 14            |

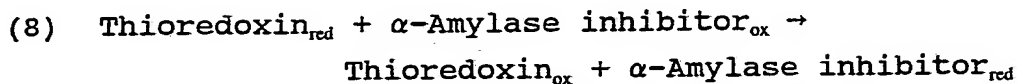
\* These values compare to the corresponding values of 40 and 550 obtained, respectively, with spinach chloroplast thioredoxin m (NADP-MDH) and thioredoxin f.

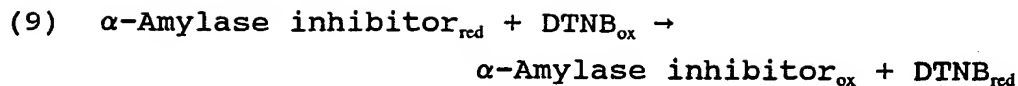
\*\* From Durum wheat

‡ From bread wheat

EXAMPLE 2DTNB Reduction Assays

A second test for thiol redox activity is the ability to catalyze the reduction of the sulfhydryl reagent, 2',5'-dithiobis(2-nitrobenzoic acid) (DTNB), measured by an increase in absorbance at 412 nm. Here, the protein assayed was reduced with NADPH via NTR and a thioredoxin. The DTNB assay proved to be effective for the  $\alpha$ -amylase inhibitors from both durum (DSG-1 and 2) and bread wheat (CM-1). When reduced by the NADP/thioredoxin system (in this case using NTR and thioredoxin from *E. coli*), either DSG-1 or DSG-2 markedly enhanced the reduction of DTNB (Fig. 4). The uppermost curve in Fig. 4 represents results obtained with either DSG-1 or -2 (NADPH  $\rightarrow$  NTR  $\rightarrow$  Thioredoxin  $\rightarrow$  DSG  $\rightarrow$  DTNB). The DTNB reduction assay was carried out with 10  $\mu$ g thioredoxin and 10  $\mu$ g NTR and 20  $\mu$ g of DSG-1 or DSG-2. CM-1 was also effective in the DTNB reduction assay, and, as with NADP-MDH activation (Table I), was detectably more active than the DSG proteins (See, Fig. 5, conditions were as in Fig. 4 except that the DSG proteins were omitted and purothionin  $\alpha$ , 20  $\mu$ g or CM-1, 20  $\mu$ g was used). The results thus confirmed the enzyme activation experiments in Example 1 and showed that the  $\alpha$ -amylase inhibitors can be reduced physiologically by the NADP/thioredoxin system. The role of the  $\alpha$ -amylase inhibitors in promoting the reduction of DTNB under these conditions is summarized in equations 7-9.





### EXAMPLE 3

#### Protein Reduction Measurements

5 The availability of monobromobimane (mBBBr) and its adaptation for use in plant systems has given a new technique for measuring the sulfhydryl groups of plant proteins (Crawford, N.A., et al. (1989), Arch. Biochem. Biophys. 271:223-239). When coupled with SDS-  
10 polyacrylamide gel electrophoresis, mBBBr can be used to quantitate the change in the sulfhydryl status of redox active proteins, even in complex mixtures. This technique was therefore applied to the inhibitor proteins to confirm their capacity for reduction by  
15 thioredoxin. Here, the test protein was reduced with thioredoxin which itself had been previously reduced with either DTT or NADPH and NTR. The mBBBr derivative of the reduced protein was then prepared, separated from other components by SDS-polyacrylamide gel  
20 electrophoresis and its reduction state was examined by fluorescence. In the experiments described below, thioredoxin from *E. coli* was found to be effective in the reduction of each of the targeted proteins. Parallel experiments revealed that thioredoxin h and  
25 calf thymus thioredoxins reduced, respectively, the proteins from seed and animal sources.

In confirmation of the enzyme activation and dye reduction experiments, DSG-1 was effectively reduced in the presence of thioredoxin. Following incubation the  
30 proteins were derivatized with mBBBr and fluorescence visualized after SDS-polyacrylamide gel electrophoresis (Fig. 6). Reduction was much less with DTT alone and was insignificant with GSH. A similar requirement for

thioredoxin was observed for the reduction of CM-1 (Fig. 7) and DSG-2 (data not shown). While the thioredoxin used in Figs. 6 and 7 was from *E. coli* similar results were obtained with wheat thioredoxin h. Thioredoxin was  
5 also required when DTT was replaced by NADPH and NTR (data not shown).

#### EXAMPLE 4

##### Thioredoxin-linked Reduction of Cystine-Rich Plant Trypsin Inhibitors

10 Whereas the major soluble cystine-rich proteins of wheat seeds can act as inhibitors of exogenous  $\alpha$ -amylases, the cystine-rich proteins of most other seeds lack this activity, and, in certain cases, act as specific inhibitors of trypsin from animal sources. While these  
15 proteins can be reduced with strong chemical reductants such as sodium borohydride (Birk, Y. (1985), Int. J. Peptide Protein Res. 25:113-131, and Birl, Y. (1976), Meth. Enzymol. 45:695-7390), there is little evidence that they can be reduced under physiological conditions.  
20 It was therefore of interest to test trypsin inhibitors for the capacity to be reduced by thioredoxin. The cystine-rich representatives tested included the soybean Bowman-Birk and corn kernel trypsin inhibitors. The results in both cases were positive: each inhibitor  
25 activated NADP-MDH (but not FBPase) when added in the presence of DTT-reduced thioredoxin (Table I) and each reduced DTNB in the presence of NADPH, NTR and thioredoxin (data not shown).

As found for the  $\alpha$ -amylase inhibitors, the thioredoxin-  
30 dependent reduction of the cystine-rich trypsin inhibitors could be directly monitored by the mBBR/SDS-polyacrylamide gel electrophoresis technique. Thus, significant reduction by DTT was observed only in the

presence of reduced thioredoxin with both the Bowman-Birk (BBTI) inhibitor (highly fluorescent fast moving band in Fig. 6) and corn kernel (CKTI) trypsin inhibitor (highly fluorescent band migrating behind thioredoxin  
5 in Fig. 7).

#### EXAMPLE 5

##### Thioredoxin-linked Reduction of Other Trypsin Inhibitors and Purothionins

In view of the finding that cystine-rich trypsin  
10 inhibitors from seeds can undergo specific reduction by thioredoxin, the question arose as to whether other types of trypsin inhibitor proteins share this property. In the course of this study, several such inhibitors -- soybean Kunitz, bovine lung aprotinin, egg white  
15 ovoinhibitor and ovomucoid trypsin inhibitors -- were tested. While the parameters tested were not as extensive as with the cystine-rich proteins described above, it was found that the other trypsin inhibitors also showed a capacity to be reduced specifically by  
20 thioredoxin as measured by both the enzyme activation and mBBBr/SDS-polyacrylamide gel electrophoresis methods. As was the case for the cystine-rich proteins described above, the trypsin inhibitors tested in this phase of the study (soybean Kunitz and animal trypsin inhibitors)  
25 activated NADP-MDH but not FBPase (Table I). Bovine lung aprotinin was an exception in that it activated FBPase more effectively than NADP-MDH. It might also be noted that aprotinin resembles certain of the seed proteins studied here in that it shows a high content  
30 of cystine (ca. 10%) (Kassel, B., et al. (1965), Biochem. Biophys. Res. Commun. 20:463-468).

The fluorescence evidence for the thioredoxin-linked reduction of one of these proteins, the Kunitz

inhibitor, is shown in Fig. 7 (highly fluorescent slow moving band). In its reduced form, the Kunitz inhibitor also yielded a fluorescent fast moving band. The nature of this lower molecular mass species is not known. Its position suggests that it could represent Bowman-Birk inhibitor present as a contaminant in the Kunitz preparation (cf. Fig. 6); however, such a component was not evident in Coomassie Blue stained SDS gels. The animal inhibitors which yielded a single fluorescent band of the expected molecular weight, also showed a thioredoxin requirement for reduction (data not shown).

In confirmation of earlier results, thioredoxin-reduced purothionin consistently activated FBPase and the type tested earlier, purothionin- $\alpha$ , failed to activate NADP-MDH (Table I) (Wada, K., et al. (1981), FEBS Lett. 124:237-240). However, in contrast to purothionin- $\alpha$  from bread wheat, two purothionins previously not examined (purothionins  $\alpha$ -1 and  $\beta$  from durum wheat) detectably activated NADP-MDH (Table I). The two durum wheat purothionins also differed in their ability to activate FBPase. The activity differences between these purothionins were unexpected in view of the strong similarity in their amino acid sequences (Jones, B.L., et al. (1977), Cereal Chem. 54:511-523) and in their ability to undergo reduction by thioredoxin. A requirement for thioredoxin was observed for the reduction of purothionin (here the  $\alpha$ -type) by the SDS-PAGE fluorescence procedure (Fig. 7).

#### EXAMPLE 6

##### Quantitation of Reduction

The above Examples demonstrate that thioredoxin reduces a variety of proteins, including  $\alpha$ -amylase, such as the CM and DSG inhibitors, and trypsin inhibitors from seed

as well as animal sources. While clear in the qualitative sense, the above results give no quantitative indication of the extent of reduction. Therefore, an experiment was conducted following the protocol of Crawford, et al. (Crawford, N.A., et al. (1989), Arch. Biochem. Biophys. 271:223-239).

As shown in Table II, the extent of reduction of the seed inhibitor proteins by the *E. coli* NADP/thioredoxin system was time-dependent and reached, depending on the protein, 15 to 48% reduction after two hours. The results, based on fluorescence emitted by the major protein component, indicate that thioredoxin acts catalytically in the reduction of the  $\alpha$ -amylase and trypsin inhibitors. The ratio of protein reduced after two hours to thioredoxin added was greater than one for both the most highly reduced protein (soybean Bowman-Birk trypsin inhibitor) and the least reduced protein (corn kernel trypsin inhibitor) -- i.e., respective ratios of 7 and 2 after a two-hour reduction period. It should be noted that the values in Table II were obtained under standard assay conditions and no attempt was made to optimize reduction by modifying those conditions.

TABLE II

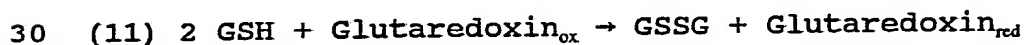
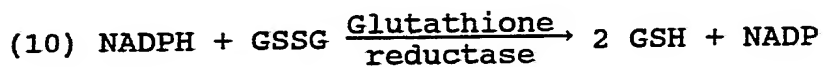
Extent of Reduction of Seed Proteins  
by the NADP/Thioredoxin System Using the  
mBBR/SDS-Polyacrylamide Gel Electrophoresis Procedure

5 The following concentrations of proteins were used  
(nmoles): thioredoxin, 0.08; NTR, 0.01; purothionin- $\beta$ ,  
1.7; DSG-1, 0.7; corn kernel trypsin inhibitor, 1.0;  
Bowman-Birk trypsin inhibitor, 1.3; and Kunitz trypsin  
inhibitor, 0.5. Except for the indicated time  
10 difference, other conditions were as in Fig. 6.

| <u>Protein</u>                   | <u>20 min</u> | <u>% Reduction After<br/>120 min</u> |
|----------------------------------|---------------|--------------------------------------|
| Purothionin- $\beta$             | 15            | 32                                   |
| 15 DSG-1                         | 22            | 38                                   |
| Corn kernel trypsin<br>inhibitor | 3             | 15                                   |
| Bowman-Birk trypsin<br>inhibitor | 25            | 48                                   |
| 20 Kunitz trypsin inhibitor      | 14            | 22                                   |

EXAMPLE 7*E. coli* Glutaredoxin as Reductant

Bacteria and animals are known to contain a thiol redox  
25 protein, glutaredoxin, that can replace thioredoxin in  
reactions such as ribonucleotide reduction (Holmgren,  
A. (1985), Annu. Rev. Biochem. 54:237-271). Glutaredoxin  
is reduced as shown in equations 10 and 11.



So far there is no evidence that glutaredoxin interacts with proteins from higher plants. This ability was tested, using glutaredoxin from *E. coli* and the seed proteins currently under study. Reduction activity was monitored by the mBBr/SDS polyacrylamide gel electrophoresis procedure coupled with densitometric scanning. It was observed that, under the conditions developed for Figs. 6 and 7, glutaredoxin could effectively replace thioredoxin in some, but not all cases. Thus, glutaredoxin was found to be active in the reduction of the following (the numbers indicate the percentage reduction relative to *E. coli* thioredoxin): DSG-1 and CM-1  $\alpha$ -amylase inhibitors (147 and 210%, respectively); corn kernel trypsin inhibitor (424%); and purothionin (82, 133, and 120% for the  $\alpha$ ,  $\alpha 1$  and  $\beta$  forms, respectively). Glutaredoxin was ineffective in the reduction of the DSG-2  $\alpha$ -amylase inhibitor and the soybean Bowman-Birk and Kunitz trypsin inhibitors. The trypsin inhibitors from animal sources also showed a mixed response to glutaredoxin. Egg white ovoinhibitor was effectively reduced (55% reduction relative to *E. coli* thioredoxin) whereas egg white ovomucoid inhibitor and bovine lung aprotinin were not affected. Significantly, as previously reported (Wolosiuk, R.A., et al. (1977), Nature 266:565-567), glutaredoxin failed to replace thioredoxin as the immediate reductant in the activation of thioredoxin-linked enzymes of chloroplasts, FBPase and NADP-MDH (data not shown).

The above Examples demonstrate that some of the enzyme inhibitor proteins tested can be reduced by glutaredoxin as well as thioredoxin. Those specific for thioredoxin include an  $\alpha$ -amylase inhibitor (DSG-2), and several trypsin inhibitors (Kunitz, Bowman-Birk, aprotinin, and ovomucoid inhibitor). Those proteins that were reduced by either thioredoxin or glutaredoxin include the purothionins, two  $\alpha$ -amylase inhibitors (DSG-1, CM-1),

a cystine-rich trypsin inhibitor from plants (corn kernel inhibitor), and a trypsin inhibitor from animals (egg white ovoinhibitor). These results raise the question of whether glutaredoxin occurs in plants.

5 Glutaredoxin was reported to be present in a green alga (Tsang, M.L.-S. (1981), Plant Physiol. 68:1098-1104) but not in higher plants.

Although the activities of the NADP-MDH and FBPase target enzymes shown in Table I are low relative to

10 those seen following activation by the physiological chloroplast proteins (thioredoxin m or f), the values shown were found repeatedly and therefore are considered to be real. It seems possible that the enzyme specificity shown by the inhibitor proteins, although

15 not relevant physiologically, reflects a particular structure achieved on reduction. It remains to be seen whether such a reduced structure is related to function within the seed or animal cell.

The physiological consequence of the thioredoxin (or

20 glutaredoxin) linked reduction event is of considerable interest as the function of the targeted proteins is unclear. The present results offer a new possibility. The finding that thioredoxin reduces a wide variety of inhibitor proteins under physiological conditions

25 suggests that, in the absence of compartmental barriers, reduction can take place within the cell.

#### EXAMPLE 8

##### Inactivation of Soybean Trypsin Inhibitor in Soybean Meal

30 The goal of this Example is to inactivate the Bowman-Birk and Kunitz trypsin inhibitors of soybeans. The following protocol applies to animal feed preparations.

To 10 g of soybean meal are added 0.2  $\mu$ g thioredoxin, 0.1  $\mu$ g NADP-thioredoxin reductase and 500 nanamoles NADPH together with 1 M Tris-HCl buffer, pH 7.9, to give 5.25 ml of 30 mM Tris-HCl. The above mixture is allowed to sit for about 30 min. at room temperature. Direct reduction of the soybean trypsin inhibitor is determined using the mBBr fluorescent labeling/SDS-polyacrylamide gel electrophoresis method previously described (Kobrehel, K., et al. (1991), J. Biol. Chem. 266:16135-16140). An analysis of the ability of the treated flour for trypsin activity is made using modifications of the insulin and BAEE (Na-benzoyl-L-arginine ethyl ester) assays (Schoellmann, G., et al. (1963), Biochemistry 252:1963; Gonias, S.L., et al. (1983), J. Biol. Chem. 258:14682). From this analysis it is determined that soybean meal so treated with the NADP/thioredoxin system does not inhibit trypsin.

#### EXAMPLE 9

##### Inactivation of $\alpha$ -Amylase Inhibitors in Cereals

To 10 g of barley malt are added 0.2  $\mu$ g thioredoxin, 0.1  $\mu$ g NADP-thioredoxin reductase and 500 nanamoles NADPH together with 1 M Tris-HCl buffer, pH 7.9, to give 5.25 ml of 30 mM Tris-HCl. The above mixture is allowed to sit for about 30 min. at room temperature. Direct reduction of the  $\alpha$ -amylase inhibitors is determined using the mBBr fluorescent labeling/SDS-polyacrylamide gel electrophoresis method previously described (Kobrehel, K., et al. (1991), J. Biol. Chem. 266:16135-16140).  $\alpha$ -Amylase activity is monitored by following the release of maltose from starch (Bernfeld, P. (1955), Methods in Enzymol. 1:149). From this analysis it is determined that barley so treated with the NADP/thioredoxin system does not inhibit  $\alpha$ -amylase.

## REDUCTION OF CEREAL PROTEINS

### Materials and Methods

#### Plant Material

Seeds and semolina of durum wheat (*Triticum durum*, Desf.  
5 cv. Monroe) were kind gifts of Dr. K. Kahn.

#### Germination of wheat seeds

Twenty to 30 seeds were placed in a plastic Petri dish  
on three layers of Whatman #1 filter paper moistened  
with 5 ml of deionized water. Germination was carried  
10 out for up to 4 days at room temperature in a dark  
chamber.

#### Reagents/Fine Chemicals

Biochemicals and lyophilized coupling enzymes were  
obtained from Sigma Chemical Co. (St. Louis, MO). *E.*  
15 *coli* thioredoxin and NTR were purchased from American  
Diagnostica, Inc. (Greenwich, CT). Wheat thioredoxin  
*h* and NTR were isolated from germ, following the  
procedures developed for spinach leaves (Florencio,  
F.J., et al. (1988), Arch. Biochem. Biophys. 266:496-  
20 507). *E. coli* glutaredoxin was a kind gift of Professor  
A. Holmgren. Reagents for SDS-polyacrylamide gel  
electrophoresis were purchased from Bio-Rad Laboratories  
(Richmond, CA). Monobromobimane (mBBBr) or Thiolite was  
obtained from Calbiochem Co. (San Diego, CA). Aluminum  
25 lactate and methyl green were products of Fluka  
Chemicals Co. (Buchs, Switzerland).

#### Gliadins and Glutenins

For isolation of insoluble storage proteins, semolina  
(0.2 g) was extracted sequentially with 1 ml of the  
30 following solutions for the indicated times at 25°C: (1)  
50 mM Tris-HCl, pH 7.5 (20 min); (2) 70% ethanol (2 hr);  
and (3) 0.1 M acetic acid (2 hr). During extraction,  
samples were placed on an electrical rotator and, in

addition, occasionally agitated with a vortex mixer. After extraction with each solvent, samples were centrifuged (12,000 rpm for 5 min.) in an Eppendorf microfuge and, supernatant fractions were saved for  
5 analysis. In between each extraction, pellets were washed with 1 ml of water, collected by centrifugation as before and the supernatant wash fractions were discarded. By convention, the fractions are designated: (1) albumin/globulin; (2) gliadin; and (3) glutenin.

10 *In vitro* mBBR labelling of proteins

Reactions were carried out in 100 mM Tris-HCl buffer, pH 7.9. As indicated, 0.7  $\mu$ g NTR and 1  $\mu$ g thioredoxin (both from *E. coli* unless specified otherwise) were added to 70  $\mu$ l of this buffer containing 1 mM NADPH and  
15 10  $\mu$ g of target protein. When thioredoxin was reduced by dithiothreitol (DTT), NADPH and NTR were omitted and DTT was added to 0.5 mM. Assays with reduced glutathione were performed similarly, but at a final concentration of 1 mM. After incubation for 20 min, 100  
20 nmoles of mBBR were added and the reaction was continued for another 15 min. To stop the reaction and derivatize excess mBBR, 10  $\mu$ l of 10% SDS and 10  $\mu$ l of 100 mM  $\beta$ -mercaptoethanol were added and the samples were then applied to the gels. For reduction by glutaredoxin, the  
25 thioredoxin and NTR were replaced by 1  $\mu$ g *E. coli* glutaredoxin, 1.4  $\mu$ g glutathione reductase (purified from spinach leaves) and 1.5 mM NADPH.

*In vivo* mBBR labelling of proteins

At the indicated times, the dry seeds or germinating  
30 seedlings (selected on the basis of similar radical length) were removed from the Petri dish and their embryos or germinated axes were removed. Five endosperms from each lot were weighed and then ground in liquid N<sub>2</sub> with a mortar and pestle. One ml of 2.0  
35 mM mBBR in 100 mM Tris-HCl, pH 7.9, buffer was added

just as the last trace of liquid N<sub>2</sub> disappeared. The thawed mixture was then ground for another minute and transferred to a microfuge tube. The volume of the suspension was adjusted to 1 ml with the appropriate mBBR or buffer solution. Protein fractions of albumin/globulin, gliadin and glutenin were extracted from endosperm of germinated seedlings as described above. The extracted protein fractions were stored at -20°C until use. A buffer control was included for each time point.

#### SDS-Polyacrylamide Gel Electrophoresis

SDS-polyacrylamide electrophoresis of the mBBR-derivatized samples was performed in 15% gels at pH 8.5 as described by Laemmli, U.K. (1970), Nature 227:680-685. Gels of 1.5 mm thickness were developed for 16 hr. at a constant current of 9 mA.

#### Native Gel Electrophoresis

To resolve the different types of gliadins, native polyacrylamide gel electrophoresis was performed in 6% gels (a procedure designed to separate gliadins into the four types) as described by Bushuk and Zillman (Bushuk, W., et al. (1978), Can. J. Plant Sci. 58:505-515) and modified for vertical slab gels by Sapirstein and Bushuk (Sapirstein, H.D., et al. (1985), Cereal Chem. 62:372-377). A gel solution in 100 ml final volume contained 6.0 g acrylamide, 0.3 g bisacrylamide, 0.024 g ascorbic acid, 0.2 mg ferrous sulfate heptahydrate and 0.25 g aluminum lactate. The pH was adjusted to 3.1 with lactic acid. The gel solution was degassed for 2 hr. on ice and then 0.5 ml of 3% hydrogen peroxide was added as a polymerization catalyst. The running buffer, also adjusted to pH 3.1 with lactic acid, contained 0.5 g aluminum lactate per liter. Duration of electrophoresis was approximately 4 hr., with a constant current of 50 mA. Electrophoresis was terminated when the solvent

front, marked with methyl green tracking dye, migrated to about 1 cm from the end of the gel.

#### mBBR Removal/Fluorescence Photography

Following electrophoresis, gels were placed in 12% (w/v) trichloroacetic acid and soaked for 4 to 6 hr. with one change of solution to fix the proteins; gels were then transferred to a solution of 40% methanol/10% acetic acid for 8 to 10 hr. to remove excess mBBR. The fluorescence of mBBR, both free and protein bound, was visualized by placing gels on a light box fitted with an ultraviolet light source (365 nm). Following removal of the excess (free) mBBR, gels were photographed with Polaroid Positive/Negative Landfilm, type 55, through a yellow Wratten gelatin filter No. 8 (cutoff=460 nm) (exposure time ranged from 25 to 60 sec at f4.5) (Crawford, N.A., et al. (1989), Arch. Biochem. Biophys. 271:223-239).

#### Protein Staining/Destaining/Photography

SDS-gels were stained with Coomassie Brilliant Blue R-250 in 40% methanol/10% acetic acid for 1 to 2 hr. and destained overnight as described before (Crawford, N.A., et al. (1989), Arch. Biochem. Biophys. 271:223-239). Aluminum lactate native gels were stained overnight in a filtered solution containing 0.1 g Coomassie Brilliant Blue R-250 (dissolved in 10 ml 95% ethanol) in 240 ml 12% trichloroacetic acid. Gels were destained overnight in 12% trichloroacetic acid (Bushuk, W., et al. (1978), Can. J. Plant Sci. 58:505-515, and Sapirstein, H.D., et al. (1985), Cereal Chem. 62:372-377).

Protein stained gels were photographed with Polaroid type 55 film to produce prints and negatives. Prints were used to determine band migration distances and loading efficiency.

The Polaroid negatives of fluorescent gels and prints of wet protein stained gels were scanned with a laser densitometer (Pharmacia-LKB UltroScan XL). Fluorescence was quantified by evaluating peak areas after  
5 integration with GelScan XL software.

#### Enzyme Assays

The following activities were determined in crude extracts with previously described methods: hexokinase (Baldus, B., et al. (1981), Phytochem. 20:1811-1814),  
10 glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase (Schnarrenberger, C., et al. (1973), Arch. Biochem. Biophys. 154:438-448), glutathione reductase, NTR and thioredoxin h (Florencio, F.J., et al. (1988), Arch. Biochem. Biophys. 266:496-507).

#### 15 Protein Determination

Protein concentrations were determined by the Bradford method (Bradford, M. (1976) Anal. Biochem. 72:248-256), with Bio-Rad reagent and bovine serum albumin as a standard.

#### 20 Subunit Molecular Weight Determination

The subunit molecular weight of gliadins and glutenins was estimated on SDS-PAGE gels by using two sets of molecular weight standards (kDa). The first set consisted of BSA (66), ovalbumin (45), soybean trypsin  
25 inhibitor (20.1), myoglobin (17), cytochrome c (12.4) and aprotinin (6.5). The other set was the BioRad Prestained Low SDS-PAGE standards: phosphorylase b (110), BSA (84), ovalbumin (47), carbonic anhydrase (33), soybean trypsin inhibitor (24) and lysozyme (16).

EXAMPLE 10Reduction of Gliadins

As a result of the pioneering contributions of Osborne and coworkers a century ago, seed proteins can be  
5 fractionated on the basis of their solubility in aqueous and organic solvents (20). In the case of wheat, preparations of endosperm (flour or semolina) are historically sequentially extracted with four solutions to yield the indicated protein fraction: (i) water,  
10 albumins; (ii) salt water, globulins; (iii) ethanol/water, gliadins; and (iv) acetic acid/water, glutenins. A wide body of evidence has shown that different proteins are enriched in each fraction. For example, the albumin and globulin  
15 fractions contain numerous enzymes, and the gliadin and glutenin fractions are in the storage proteins required for germination.

Examples 1, 4 and 5 above describe a number of water soluble seed proteins (albumins/globulins, e.g.,  
20  $\alpha$ -amylase inhibitors, cystine-rich trypsin inhibitors, other trypsin inhibitors and thionines) that are reduced by the NADP/thioredoxin system, derived either from the seed itself or *E. coli*. The ability of the system to reduce insoluble storage proteins from wheat seeds,  
25 viz., representatives of the gliadin and glutenin fractions, is described below. Following incubation with the indicated additions, the gliadin proteins were derivatized with mBBR and fluorescence was visualized after SDS-polyacrylamide gel electrophoresis. The lanes  
30 in Fig. 8 were as follows: 1. Control: no addition. 2. GSH/GR/NADPH: reduced glutathione, glutathione reductase (from spinach leaves) and NADPH. 3. NGS: NADPH, reduced glutathione, glutathione reductase (from spinach leaves) and glutaredoxin (from *E. coli*). 4. NTS:  
35 NADPH, NTR, and thioredoxin (both proteins from *E.*

*coli*). 5. MET/T(Ec):  $\beta$ -mercaptoethanol and thioredoxin (*E. coli*). 6. DTT. 7. DTT/T(Ec): DTT and thioredoxin (*E. coli*). 8. DTT/T(W): Same as 7 except with wheat thioredoxin h. 9. NGS,-Gliadin: same as 3 except without the gliadin protein fraction. 10. NTS,-Gliadin: same as 4 except without the gliadin protein fraction. Based on its reactivity with mBBR, the gliadin fraction was extensively reduced by thioredoxin (Fig. 8). The major members undergoing reduction showed a Mr ranging from 25 to 45 kDa. As seen in Examples 1, 4 and 5 with the seed  $\alpha$ -amylase and trypsin inhibitor proteins, the gliadins were reduced by either native h or *E. coli* type thioredoxin (both homogeneous); NADPH (and NTR) or DTT could serve as the reductant for thioredoxin. Much less extensive reduction was observed with glutathione and glutaredoxin--a protein able to replace thioredoxin in certain *E. coli* and mammalian enzyme systems, but not known to occur in higher plants.

The gliadin fraction is made up of four different protein types, designated  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\omega$ , which can be separated by native polyacrylamide gel electrophoresis under acidic conditions (Bushuk, W., et al. (1978), Can. J. Plant Sci. 58:505-515; Kasarda, D.D., et al. (1976), Adv. Cer. Sci. Tech. 1:158-236; Sapirstein, H.D., et al. (1985), Cereal Chem. 62:372-377; and Tatham, A.S., et al. (1990), Adv. Cer. Sci. Tech. 10:1-78). Except for the  $\omega$  gliadins, each species contains cystine (S-S) groups and thus has the potential for reduction by thioredoxin. In this study, following incubation with the indicated additions, proteins were derivatized with mBBR, and fluorescence was visualized after acidic-polyacrylamide gel electrophoresis. The lanes in Fig. 9 were as follows: 1. Control: no addition. 2. GSH: reduced glutathione. 3. GSH/GR/NADPH: reduced glutathione, glutathione reductase (from spinach leaves) and NADPH. 4. NGS: NADPH, reduced glutathione,

glutathione reductase (from spinach leaves) and glutaredoxin (from *E. coli*). 5. NGS+NTS: combination of 4 and 6. 6. NTS: NADPH, NTR, and thioredoxin (both proteins from *E. coli*). 7. MET/T(Ec):  $\beta$ -mercaptoethanol and thioredoxin (*E. coli*). 8. DTT/T(Ec): DTT and thioredoxin (*E. coli*). 9. NTS(-T): same as 6 except without thioredoxin. 10. NGS+NTS,-Gliadin: same as 5 except without the gliadin fraction.

When the thioredoxin-reduced gliadin fraction was subjected to native gel electrophoresis, the proteins found to be most specifically reduced by thioredoxin were recovered in the  $\alpha$  fraction (See, Fig. 9). There was active reduction of the  $\beta$  and  $\gamma$  gliadins, but as evident from the densitometer results summarized in Table III, the reduction within these groups was nonspecific, i.e., relatively high levels of reduction were also achieved with glutathione and glutaredoxin. There was especially strong reduction of the  $\gamma$  gliadins by DTT-reduced thioredoxin (Fig. 9). As anticipated, there was no reduction of the  $\omega$  gliadins. The evidence indicates that thioredoxin (either native h or *E. coli*) specifically reduces certain of the gliadins, especially the  $\alpha$  type.

#### EXAMPLE 11

##### Reduction of Glutenins

The remaining group of seed proteins to be tested for a response to thioredoxin -- the glutenins -- while the least water soluble, are perhaps of greatest interest. The glutenins have attracted attention over the years because of their importance for the cooking quality of flour and semolina (MacRitchie, F., et al. (1990), Adv. Cer. Sci. Tech. 10:79-145). Testing the capability of

thioredoxin to reduce the proteins of this group was, therefore, a primary goal of the current investigation.

TABLE III

Reductant Specificity of  
the Different Types of Gliadins

The area under  $\alpha$ ,  $\beta$ ,  $\gamma$  and aggregate peaks following reduction by the NADP/thioredoxin system were: 4.33, 8.60, 5.67 and 0.74 Absorbance units times millimeters, respectively. These combined areas were about 65% of those observed when thioredoxin was reduced by DTT. Reaction conditions as in Fig. 9.

|    | <u>Reductant</u> | <u>Gliadin, % Relative Reduction</u> |         |          |            |
|----|------------------|--------------------------------------|---------|----------|------------|
|    |                  | $\alpha$                             | $\beta$ | $\gamma$ | Aggregate* |
|    | None             | 22.4                                 | 30.4    | 24.3     | 29.2       |
| 15 | Glutathione      | 36.4                                 | 68.1    | 60.6     | 60.1       |
|    | Glutaredoxin     | 43.5                                 | 83.3    | 79.7     | 61.5       |
|    | Thioredoxin      | 100.0                                | 100.0   | 100.0    | 100.0      |

\*Proteins not entering the gel

As seen in Fig. 10 (treatments were as in Example 10, Fig. 8), several glutenins were reduced specifically by thioredoxin. The most extensive reduction was observed in the low molecular mass range (30 - 55 kDa). The reduction observed in the higher molecular mass range was less pronounced, but still obvious--especially in the 100 kDa region and above. Though not shown reduction may also occur in the 130 kDa range. Like the gliadins, certain of the glutenins were appreciably reduced by glutathione and glutaredoxin. However, in all cases, reduction was greater with thioredoxin and, in some cases, specific to thioredoxin (Table IV, note proteins in the 30 - 40 and 60 - 110 kDa range). As observed with the other wheat proteins tested, both the native h anal *E. coli* thioredoxins were active and could

be reduced with either NADPH and the corresponding NTR or with DTT. Thus as found for the gliadins, certain glutenins were reduced *in vitro* specifically by thioredoxin, whereas others were also reduced, albeit less effectively, by glutathione and glutaredoxin.

TABLE IV

Reductant Specificity of Glutenins

Reaction conditions as in Fig. 3.

| 10 | <u>Reductant</u> | <u>Glutenin, % Relative Reduction*</u> |           |           |
|----|------------------|--|-----------|-----------|
|    |                  | 60-110 kDa                             | 40-60 kDa | 30-40 kDa |
|    | None             | 8                                      | 23        | 16        |
|    | Glutathione      | 31                                     | 51        | 29        |
|    | Glutaredoxin     | 50                                     | 72        | 40        |
|    | Thioredoxin*     | 100                                    | 100       | 100       |

15 \*Area under the three molecular weight classes (from high to low) following reduction by the NADP/thioredoxin system were: 1.5, 5.67 and 5.04 Absorbance units times millimeters, respectively.

EXAMPLE 1220 *In vivo* Reduction Experiments

The above Example demonstrates that thioredoxin specifically reduces components of the wheat gliadin and glutenin fractions when tested *in vitro*. The results, however, provide no indication as to whether these proteins are reduced *in vivo* during germination -- a question that, to our knowledge, had not been previously addressed (Shutov, A.D., et al. (1987), Phytochem. 26:1557-1566).

To answer this question, we applied the mBBR/SDS-PAGE technique was applied to monitor the reduction status of proteins in the germinating seed. We observed that reduction of components in the Osborne fractions increased progressively with time and reached a peak

after 2 to 3 days germination (Fig. 11). The observed increase in reduction ranged from 2-fold with the gliadins, to 3-fold with the albumin/globulins and 5-fold with the glutenins. The results suggest that, while representatives of the major wheat protein groups were reduced during germination, the net redox change was greatest with the glutenins.

Although providing new evidence that the seed storage proteins undergo reduction during germination, the results of Fig. 11 give no indication as to how reduction is accomplished, i.e., by glutathione or thioredoxin. To gain information on this point, the *in vivo* reduction levels of the principal thioredoxin-linked gliadins (30 - 50 kDa) and glutenins (30 - 40, 40 - 60 kDa) was compared with the reduction determined from *in vitro* measurements (cf. Fig. 8 and Table IV). For this purpose, the ratio of fluorescence to Coomassie stained protein observed *in vivo* during germination and *in vitro* with the appropriate enzyme reduction system was calculated. The results shown in Fig. 12 (principal thioredoxin linked gliadins were those in the Mr range from 25 to 45 kDa, see Fig. 8, and glutenins were those in the Mr range from 30 to 60 kDa, see Fig. 10) suggest that, while glutathione could account for a significant part of the *in vivo* reduction of the gliadin fraction (up to 90%), this was not the case with the glutenins whose reduction seemed to require thioredoxin. The level of reduction that could be ascribed to glutathione (or glutaredoxin) was insufficient to account for the levels of reduced glutenin measured in the germinating seed.

EXAMPLE 13Enzyme Measurements

The source of NADPH needed for the NTR linked reduction of thioredoxin h was also investigated. Semolina was  
5 analyzed for enzymes that function in the generation of NADPH in other systems, notably dehydrogenases of the oxidative phosphate pathway. The results summarized in Table V confirm earlier evidence that endosperm extracts contain the enzymes needed to generate NADPH from  
10 glucose via this pathway: hexokinase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase (Tatham, A.S., et al. (1990), Adv. Cer. Sci. Tech. 10:1-78). It is noteworthy that the glucose 6-phosphate dehydrogenase activity seen in Table V was  
15 insensitive to reduced thioredoxin (data not shown). In this respect the endosperm enzyme resembles its cytosolic rather than its chloroplast counterpart from leaves (Fickenscher, K., et al. (1986), Arch. Biochem. Biophys. 247:393-402; Buchanan, B.B. (1991), Arch. Biochem. Biophys. 288:1-9; Scheibe, R., et al. (1990),  
20 Arch. Biochem. Biophys. 274:290-297).

As anticipated from earlier results with flour (Johnson, T.C., et al. (1987), Planta 171:321-331; Suske, G., et al. (1979), Z. Naturforsch. C 34:214-221), semolina also  
25 contained thioredoxin h and NTR (Table V). Interestingly, based on activity measurements, NTR appeared to be a rate-limiting component in preparations from the cultivar examined.

TABLE V

Activities of Enzymes Effecting  
the Reduction of Thioredoxin h in Semolina  
(Glucose→Glu-6-P→6-P-Gluconate→NADP→Thioredoxin h)

| 5  | <u>Protein</u>              | <u>Activity</u><br><u>(nkat/mg protein)</u> |
|----|-----------------------------|---|
|    | Hexokinase                  | 0.28  |
|    | Glucose-6-P dehydrogenase   | 0.45  |
|    | 6-P-Gluconate dehydrogenase | 0.39  |
| 10 | NTR                         | 0.06  |
|    | Thioredoxin <u>h</u>        | 0.35  |

The present results suggest that thioredoxin h functions as a signal to enhance metabolic processes associated with the germination of wheat seeds. Following its  
15 reduction by NTR and NADPH (generated via the oxidative pentose phosphate pathway), thioredoxin h appears to function not only in the activation of enzymes, but also in the mobilization of storage proteins.

EXAMPLE 1420 Improvement of Dough Quality

Dough quality was improved by reducing the flour proteins using the NADP/thioredoxin system. Reduced thioredoxin specifically breaks sulfur-sulfur bonds that cross-link different parts of a protein and stabilize  
25 its folded shape. When these cross-links are cut the protein can unfold and link up with other proteins in bread, creating an interlocking lattice that forms the elastic network of dough. The dough rises because the network traps carbon dioxide produced by yeast in the  
30 fermenting process. It is proposed that the reduced thioredoxin activated the gliadins and glutenins in flour letting them recombine in a way that strengthened the dough (Fig. 13). Reduced thioredoxin strengthened

the protein network formed during dough making. For these tests, namely those shown in Fig. 14(c) and Fig. 15(d) (using 10 gm of either intermediate quality wheat flour obtained from a local miller in Montpellier, France (Fig. 14), or poor quality wheat also obtained from a local miller in Montpellier, France (Fig. 15), this poor quality wheat being mainly of the Apollo cultivar), 0.2  $\mu$ g *E. coli* thioredoxin, 0.1  $\mu$ g *E. coli* NADP-thioredoxin reductase and 500 nanomoles NADPH were added together with 1 M Tris-HCl, pH 7.9 buffer to give 5.25 ml of a 30 mM Tris-HCl enzyme system mixture. The reaction was carried out by mixing the enzyme system mixture with the 10 gm of the flour in a micro-farinograph at 30°C. As seen in Figs. 14 and 15, the resulting farinograph measurements showed a strengthening of the dough by the added NADP/thioredoxin system. With a flour of poor quality, as in Fig. 15(d), the farinograph reading was stable for at least 4 min. after the dough was formed in the presence of the reduction system, whereas the reading dropped immediately after dough formation in the control without this addition (see Fig. 15(a)). The improving effect was persistent and was maintained throughout the run. Expressed another way, the micro-farinograph reading is 375 Brabender units, 7 min. after dough formation with the poor quality wheat control (no added enzyme system) versus 450 Brabender units for the same poor quality wheat treated with components of the NADP/thioredoxin system (NADPH, thioredoxin and NADP-thioredoxin reductase).

Another farinograph study was carried out as above with 10 gm of Apollo flour only the concentration of NADPH was 500  $\mu$ moles instead of nanomoles. As shown in the farinograph measurements in Fig. 16 this amount of NADPH also resulted in a definite improvement in the quality of the dough.

Higher farinograph measurements of dough correspond to improved dough strength and improved baked good characteristics such as better crumb quality, improved texture and higher loaf volume. Also, based on *in vivo* analyses with the isolated proteins, the native wheat seed NADP/thioredoxin system will also be effective in strengthening the dough.

For purposes of baking and other aspects of this invention, ranges of about 0.1 to 3.0  $\mu\text{g}$  of a thioredoxin (preferably *E. coli* or thioredoxin *h*) and from about 0.1 to 2.0  $\mu\text{g}$  reductase and about 30 to 500 nanomoles of NADPH are added for about every 10 gm of flour. The optimal levels of thioredoxin and reductase depend on flour quality. In general, the higher the flour quality, the higher the level of thioredoxin and reductase required. Thioredoxin can also be reduced by lipoic acid instead of by the NADPH/NADP-thioredoxin reductase reduction system. The other dough ingredients such as milk or water are then added. However, the liquid may first be added to the NTR/thioredoxin system and then added to the flour. It is preferred that yeast for purposes of leavening be added after the reduced thioredoxin has had a chance to reduce the storage proteins. The dough is then treated as a regular dough proofed, shaped, etc. and baked.

NADPH can be replaced in this Example as well as in the following Examples with an NADPH generator such as one consisting of 100  $\mu\text{M}$  glucose 6-phosphate, 100  $\mu\text{M}$  NADP and 0.05 units (0.2  $\mu\text{gram}$ ) glucose 6-phosphate dehydrogenase from a source such as yeast. The NADPH generator is added together with thioredoxin and NADP-thioredoxin reductase at the start of the dough making process.

Fig. 17(c) shows the higher farinograph measurement obtained when 10 gm of Apollo cultivar (CV) wheat are reacted with 20  $\mu$ l NADP (25 mM), 20  $\mu$ l G6P (25 mM), 0.25  $\mu$ g G6PDase, 0.1  $\mu$ g NTR and 0.2  $\mu$ g thioredoxin h  
5 contained in 4.25 ml H<sub>2</sub>O and 0.90 ml Tris-HCl (30 mM, pH 7.9). Fig. 17(b) shows that a higher farinograph measurement is also obtained when 10 gm of Apollo wheat are reacted with the same reaction mixture as the mixture resulting in Fig. 17(c) but without any NTR or  
10 thioredoxin.

#### EXAMPLE 15

##### Wheat Bread Baking Studies

The baking tests were carried out by using a computer monitored PANASONIC baking apparatus.

#### 15 Composition of bread:

##### Control:

|    |              |   |
|----|--------------|---|
|    | Flour*:      | 200 gm (dry)  |
|    | Water:       | 70% hydratation   |
|    | Salt (NaCl): | 5.3 g   |
| 20 | Yeast:       | 4.8 g ( <i>Saccharomyces cerevisiae</i> ,<br>SafInstant) (dry yeast powder) |

\* Flour samples were obtained from pure bread wheat cultivars having contrasting baking quality (including animal feed grade and other grades having from poor to  
25 good baking quality).

##### Assays:

The dough for the assays contained all the components of the control plus as indicated varying amounts of the NADP Thioredoxin System (NTS) and/or the NADP generating  
30 System.

Experimental conditions

- Flour and salt are weighed and mixed
- The volume of water needed to reach a hydration of 70% was put into the baking pan.
- 5    -- The mixture of flour and salt was added to the water and the baking program monitored by the computer was started. The complete program lasted 3 hrs 9 min and 7 secs.
- 10    -- In the case of the assays, enzyme system components are added to the water before the addition of the flour-salt mixture.
- Yeast was added automatically after mixing for 20 min and 3 secs.

The program monitoring the Panasonic apparatus was:

| 15 | <u>Segments</u> | <u>Duration</u> | <u>Mixing<br/>Conditions</u> | <u>Heating</u> |
|----|-----------------|-----------------|------------------------------|----------------|
|    | Mixing          | 00:00:03        | T1                           | off            |
|    | Mixing          | 00:05:00        | T2                           | off            |
|    | Mixing          | 00:05:00        | T1                           | off            |
| 20 | Rest            | 00:10:00        | TO                           | off            |
|    | Mixing          | 00:17:00        | T2                           | off            |
|    | Mixing          | 00:07:00        | T1                           | off            |
|    | Rest            | 00:30:00        | TO                           | to reach 32°C  |
|    | Mixing          | 00:00:04        | T1                           | 32°C           |
| 25 | Rest            | 01:15:00        | TO                           | 32°C           |
|    | Baking          | 00:14:00        | TO                           | to reach 180°C |
|    | Baking          | 00:26:00        | TO                           | 180°C          |

- Mixing Conditions: TO = no mixing (motor at rest)  
                           T1 = normal mixing  
                           T2 = alternately 3 second mixing,  
   3 second rest
- 30

Bread loaf volume was determined at the end of the baking, when bread loaves reached room temperature.

Cultivar THESEE Assay

- 35    The french wheat cultivar Thesesee is classified as having good breadmaking quality. Table VI below sets forth the results of the assay.

TABLE VI

|       |  | NADPH<br>( $\mu$ moles) | NTR<br>( $\mu$ g) | Th<br>( $\mu$ g) | <u>Loaf Volume</u><br>(cm3)    | Relative<br>Units   |
|-------|--|-------------------------|-------------------|------------------|--------------------------------|---------------------|
| 5     | Control  | 0                       | 0                 | 0                | 1690                           | 100                 |
|       | Samples  | 6.0                     | 30                | 60               | 1810                           | 107                 |
|       |  | 6.0                     | 30                | 0                | 1725                           | 102                 |
|       |  | 6.0                     | 0                 | 60               | 1720                           | 102                 |
|       |  | 6.0                     | 0                 | 0                | 1550                           | 92                  |
| 10    |  | 0                       | 30                | 60               | 1800                           | 107                 |
| <hr/> |  |                         |                   |                  |                                |                     |
|       | *NADPH<br>Generating syst.                                       |                         | 30                | 60               | 1620                           | 96                  |
| 15    | *NADPH<br>Generating<br>syst. plus<br>ATP, glucose               |                         | 30                | 60               | 1630                           | 96                  |
| <hr/> |  |                         |                   |                  |                                |                     |
| 20    | NTR and<br>Th from<br>yeast                                      | 6.0                     | 9.4               | 20               | 1750                           | 104                 |
| <hr/> |  |                         |                   |                  |                                |                     |
| 25    | *Composition of the NADPH generating system, ATP and<br>glucose. |                         |                   |                  |                                | <u>Volume Added</u> |
|       | NADP, 25 mMolar  |                         |                   |                  | 700 $\mu$ l (17.5 $\mu$ moles) |                     |
|       | Glucose-6-phosphate, 25 mMolar                                   |                         |                   |                  | 700 $\mu$ l (17.5 $\mu$ moles) |                     |
|       | Glucose-6-phosphate<br>dehydrogenase (50 $\mu$ g/ml)             |                         |                   |                  | 175 $\mu$ l (8.75 $\mu$ g)     |                     |
| 30    | ATP, 25 mMolar   |                         |                   |                  | 700 $\mu$ l (17.5 $\mu$ moles) |                     |
|       | Glucose, 25 mMolar   |                         |                   |                  | 700 $\mu$ l (17.5 $\mu$ moles) |                     |

As shown in Table VI, an increased loaf volume was obtained when the complete NTS at concentrations of 6.0  $\mu$ moles NADPH, 30  $\mu$ g NTR and 60  $\mu$ g Th was used to bake loaves from 200 g of Thesee flour with the amounts and conditions described above in this Example. Unless otherwise stated, the NTR and thioredoxin (th) were from

*E. coli*. No similar increase occurred when the generating system was used or when either NTR or Th were omitted. Also no significant effect on loaf volume occurred when amounts of the components in the system were about half or less than half of the amounts of above.

#### Cultivar APOLLO Assay

This French wheat cultivar is classified as having poor breadmaking quality. The NTR and thioredoxin used in this assay were from *E. coli*. Table VII below sets forth the results of this assay using 200 gm of Apollo flour. Again unless otherwise stated the amounts and conditions are those described above at the beginning of the Example.

TABLE VII

|    |                                    | <u>NADPH</u><br><u>(<math>\mu</math>moles)</u> | <u>NTR</u><br><u>(<math>\mu</math>g)</u> | <u>Th</u><br><u>(<math>\mu</math>g)</u> | <u>Loaf Volume</u> |                                 |
|----|------------------------------------|--|--|---|--------------------|---------------------------------|
|    |                                    |  |  |   | <u>(cm3)</u>       | <u>Relative</u><br><u>Units</u> |
| 5  | Control                            | 0  | 0  | 0                                       | 1400               | 100                             |
|    | Samples                            | 6.0  | 30                                       | 60                                      | 1475               | 105                             |
|    | *NADPH<br>Generating<br>syst. plus |  |  |   |                    |                                 |
| 10 | ATP, glucose                       |  | 30                                       | 60                                      | 1530               | 109                             |
|    | *NADPH<br>Generating<br>syst. plus |  |  |   |                    |                                 |
|    | ATP, glucose                       |  | 0  | 0                                       | 1430               | 102                             |
| 15 | *NADPH<br>Generating<br>syst.      |  | 6  | 0                                       | 1430               | 102                             |
|    | *NADPH<br>Generating<br>syst.      |  |  |   |                    |                                 |
| 20 |                                    |  | 6  | 7                                       | 1440               | 103                             |

\*The composition of the generating system, ATP and glucose is as in Table VI.

#### Cultivar ARBON Assay

25 The French wheat cultivar Arbon is used for feed and is classified as non suitable for breadmaking. Tables VIII and IX below show that an improved bread loaf volume can be obtained from Arbon using the NTS or NADPH and NTR with the dough components and conditions described at

30 the beginning of the Example. The amounts of NTR, thioredoxin, NADPH and the NADPH generating system components used in the assay are set forth in Tables VIII and IX. The improvement in Arbon bread quality using the complete NTS as set forth in Table IX is also

clearly seen in the photographs shown in Figs. 18-22 and 23(a).

TABLE VIII

| 5  |         | <u>NADPH</u><br><u>(<math>\mu</math>moles)</u> | <u>NTR</u><br><u>(<math>\mu</math>g)</u> | <u>Th</u><br><u>(<math>\mu</math>g)</u> | <u>Loaf Volume</u>                      |
|----|---------|--|--|---|---|
|    |         |  |  |   | <u>(cm<sup>3</sup>)</u>                 |
|    | Control | 0  | 0  | 0                                       | 1350                                    |
|    | Samples | 0.1-0.6  | 3-4                                      | 3-4                                     | up to 20%<br>higher than<br>the control |
| 10 |         | >2.0   | >20                                      | >20                                     | less than<br>the control                |

TABLE IX

| 15 |   | <u>Loaf Volume</u>      | <u>Relative</u> |
|----|---|-------------------------|-----------------|
|    |   | <u>(cm<sup>3</sup>)</u> | <u>Units</u>    |
|    | <u>Treatment</u>                              |                         |                 |
|    | Complete NTS                                  | 1650                    | 122             |
|    | minus Thioredoxin                             | 1690                    | 125             |
| 20 | minus NTR                                     | 1520                    | 113             |
|    | minus Thioredoxin, NTR                        | 1540                    | 114             |
|    | minus NADPH                                   | 1440                    | 107             |
|    | minus NADPH, plus *NADPH<br>generating system | 1560                    | 116             |
| 25 | minus NTS (control)                           | 1350                    | 100             |

---

NADPH, 0.6  $\mu$ moles  
Thioredoxin, 3.5  $\mu$ g  
NTR, 3  $\mu$ g

30 \*Generating System:  
3.5  $\mu$ moles NADP  
3.5  $\mu$ moles glucose-6-phosphate  
1.75  $\mu$ g glucose-6-phosphate dehydrogenase

EXAMPLE 16Triticale Bread Baking Study

Triticale is a wheat/rye hybrid and is generally used for chicken feed. It is more nutritious than wheat but is not generally considered appropriate for breadmaking, especially in the more developed nations. The effect of the NTS system and variations thereof on loaves baked from Triticale flour was consequently studied. Unless otherwise stated, the baking conditions and dough ingredient were as described for wheat flour in Example 15. As shown in Table X there is an improvement in loaf volume when the triticale dough contained thioredoxin, NTR and the NADPH generating system in the amounts set forth in that Table. However, no corresponding improvement was seen when the NTS (*i.e.*, thioredoxin, NTR and NADPH) was used. Fig. 24 shows that an improvement in the texture of the bread also occurred when NTR, Th and the NADPH generating system as set forth in Table X were used. The loaf on the right in Fig. 24 is the control.

TABLE X

Effect of the NADP/Thioredoxin System (NTS)  
on Loaves Baked from Triticale Flour (cv. Juan)

| 5     | <u>Treatment</u>                              | <u>Loaf Volume</u>      | <u>Relative</u> |
|-------|---|-------------------------|-----------------|
|       |   | <u>(cm<sup>3</sup>)</u> | <u>Units</u>    |
|       | Complete NTS                                  | 1230                    | 94              |
|       | minus NTS (control)                           | 1310                    | 100             |
| 10    | minus NADPH, plus *NADPH<br>generating system | 1390                    | 106             |
| <hr/> |   |                         |                 |
|       | NADPH, 0.6 $\mu$ moles                        |                         |                 |
|       | Thioredoxin, 3.5 $\mu$ g                      |                         |                 |
| 15    | NTR, 3.0 $\mu$ g                              |                         |                 |
|       | Generating System:                            |                         |                 |
|       | 4.5 $\mu$ moles NADP                          |                         |                 |
|       | 4.5 $\mu$ moles glucose-6-phosphate           |                         |                 |
|       | 4.5 $\mu$ g glucose-6-phosphate dehydrogenase |                         |                 |

20

EXAMPLE 17

The effect of the NADPH/thioredoxin system on flour from sorghum, corn and rice was also determined. The baking conditions were as described for wheat flour in Example 15. The amounts of the components of the NTS as used  
25 in this assay were as follows: 8  $\mu$ moles NADPH, 40.5  $\mu$ g NTR and 54  $\mu$ g thioredoxin. Both the thioredoxin and NTR were from *E. coli*. The results of this assay are shown in Fig. 25 and also in Fig. 23(b). As shown in these figures the breads containing the NTS, especially corn  
30 and sorghum exhibited improved texture and stability.

EXAMPLE 18Reduction of Ethanol-Soluble and  
Myristate-Soluble Storage Proteins from Triticale,  
Rye, Barley, Oat, Rice, Sorghum, Corn and Teff

5 Unless otherwise stated, the materials and methods used in this Example are according to those set forth above in the section titled "Reduction of Cereal Proteins, Materials and Methods."

Triticale, Rye, Barley, Oat and Teff

10 The reactions were carried out in 30 mM Tris-HCl buffer, pH 7.9. As indicated, 0.7  $\mu$ g of NTR and 1  $\mu$ g of thioredoxin from *E. coli* or 2  $\mu$ g of thioredoxin from yeast, as identified, were added to 70  $\mu$ L of this buffer containing 1 mM NADPH and 25 to 30  $\mu$ g of extracted  
15 storage protein. The ethanol extracted storage proteins were obtained by using 50 ml of 70% ethanol for every 10 gm of flour and extracting for 2 hr. In the case of teff, 200 mg of ground seeds were extracted. The myristate extracted proteins were obtained by extracting  
20 1 gm of flour with 8 mg sodium myristate in 5 ml of distilled H<sub>2</sub>O for 2 hrs. The combination of NADPH, NTR and thioredoxin is known as the NADP/thioredoxin system (NTS). As indicated, glutathione (GSH), 2.5 mM, was added as reductant in either the absence (GSH) or  
25 presence of 1.5 mM NADPH and 1.4  $\mu$ g of spinach leaf glutathione reductase (GR/GSH/NADPH). After incubation for 20 min, 100 nmol of mBBr was added and the reaction was continued for another 15 min. To stop the reaction and derivatize excess mBBr, 10  $\mu$ L of 10% SDS and 10  $\mu$ L  
30 of 100 mM 2-mercaptoethanol were added, and the samples were then applied to the gels. The procedure for SDS-polyacrylamide gel electrophoresis was as described by N. A. Crawford, et al. (1989 Arch. Biochem. Biophys. 271:223-239).

Rice, Sorghum and Corn

The reactions were carried out in 30 mM Tris-HCl buffer, pH 7.9. When proteins were reduced by thioredoxin, the following were added to 70  $\mu$ L of buffer: 1.2 mM NADPH, 10 to 30  $\mu$ g of seed protein fraction, 0.5  $\mu$ g *E. coli* NTR and 1  $\mu$ g *E. coli* thioredoxin. For reduction with glutathione, thioredoxin and NTR were replaced with 2.5 mM reduced glutathione and 1  $\mu$ g glutathione reductase (baker's yeast, Sigma Chemical Co.). For reduction with dithiothreitol, NADPH, thioredoxin, and NTR were omitted and 0.5 mM dithiothreitol was added. In all cases, incubation time was 20 min. Then 10  $\mu$ l of a 10 mM mBBR solution was added and the reaction continued for an additional 15 min. To stop the reaction and derivatize excess mBBR, 10  $\mu$ l of 10% SDS and 10  $\mu$ l of 100 mM 2-mercaptoethanol were added and the samples applied to the gels. In each case, to obtain the extracted protein, 1 g ground seeds was extracted with 8 mg of sodium myristate in 5 ml distilled water. With the exception of the initial redox state determination of the proteins, samples were extracted for 2 hr at 22°C and then centrifuged 20 min at 16,000 rpm prior to the addition of the mBBR. With the initial redox state determination, the mBBR was added under a nitrogen atmosphere along with the myristate followed by extraction.

Figs. 26-30 represent pictures of the gels for the reduction studies of myristate-extracted proteins from flour of oat, triticale, rye, barley and teff. Buffer and ethanol-extracted proteins are also shown for teff in Fig. 30. In all of the studies represented by Figs. 26-30, the flour was first extracted with buffer, 50 mM Tris-HCl, pH 7.5 for 20 min. and then with 70% ethanol for 2 hr. Also shown are pictures of the gels for the myristate-extracted proteins from corn, sorghum and rice (Figs. 31 and 32). With corn, sorghum and rice, the

ground seeds were extracted only with myristate. Therefore, with corn, sorghum and rice, the myristate extract represents total protein, whereas with oat, triticale, rye, barley and teff, the myristate extract  
5 represents only the glutenin-equivalent fractions since these flours had been previously extracted with buffer and ethanol. The results, depicted in the gels in Figs. 26-30, show that the NTS is most effective, as compared to GSH or GSH/GR/NADPH, with myristate-extracted  
10 (glutenin-equivalent) proteins from oat, triticale, rye, barley and teff. The NTS is also most effective with the total proteins from rice (Figs. 31 and 32). Reduced glutathione is more effective with the total proteins from corn and sorghum (Figs. 31 and 32).

15 Conclusions from Figs. 31 and 32  
(corn, sorghum and rice)

As depicted in Fig. 31 in treatment (1), extraction with myristate in the presence of mBBR was carried out under a nitrogen atmosphere; in treatment (2), to the  
20 myristate extracted proteins mBBR was added without prior reduction of the proteins; in treatment (3), the myristate extracted proteins were reduced by the NADP/thioredoxin system (NTS); in treatment (4) the myristate extracted proteins were reduced by NADPH,  
25 glutathione and glutathione reductase. As depicted in Fig. 32, treatment (1) is like treatment (2) in Fig. 31; in treatment (2) the seeds were extracted with myristate in the presence of mBBR under nitrogen; in treatment (3), seeds were extracted with myristate and reduced by  
30 the NTS and then mBBR was added; and in treatment (4) conditions as in (3) except that proteins were reduced by DTT. Treatment (1) in Fig. 31 and treatment (2) in Fig. 32 show the initial redox state of the proteins in the grains. For all three cereals, the proteins in the  
35 seed are highly reduced. If extracted in air, the proteins become oxidized especially the sorghum and

rice. The oxidized proteins can be re-reduced, maximally with NTS in all cases. With rice, the reduction is relatively specific for thioredoxin; with corn, glutathione is as effective as thioredoxin and  
5 with sorghum glutathione is slightly more effective than thioredoxin. Dithiothreitol showed varying effectiveness as a reductant. These experiments demonstrate that the storage proteins of these cereals are less specific than  
10 in the case of wheat and suggest that thioredoxin should be tested both in the presence and absence of glutathione when attempting to construct a dough network.

Figs. 33 and 34 represent pictures of the gels resulting from the reduction studies of wheat glutenins and  
15 gliadins, respectively, by a yeast NADP/thioredoxin system. The glutenins were obtained by using 50 ml of 0.1 M acetic acid for every 10 gm of flour and extracting for 2 hr. The gliadins were obtained by using 50 ml of 70% ethanol for every 10 gm of flour and  
20 extracting for 2 hr. The experiment shows that the yeast system is highly active in reducing the two major groups of wheat storage proteins.

Figs. 35-38 represent pictures of gels for the reduction of ethanol-extracted proteins from flour of triticale,  
25 rye, oat and barley, respectively. The results show that the NTS is most effective with the ethanol-extracted proteins from triticale, rye and oat. The ethanol-extracted barley proteins are reduced in the control and thioredoxin or glutathione has little  
30 effect.

EXAMPLE 19Effect of Thioredoxin-linked Reduction on the  
Activity and Stability of the Kunitz and  
Bowman-Birk Soybean Trypsin Inhibitor Proteins5                   Materials and MethodsPlant materials

Durum wheat (*Triticum durum*, Desf. cv. Monröe) was a kind gift of Dr. K. Kahn. Wheat germ was obtained from Sigma Chemical Co. (St. Louis, MO).

10   Chemicals and Enzymes

Reagents for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were obtained from Bio-Rad Laboratories (Richmond, CA), and DTT was from Boehringer Mannheim Biochemicals (Indianapolis, IN). L-1-  
15   Tosylamide-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (type XIII, T8640), subtilisin (type VIII: bacterial subtilisin Carlsberg, P5380), KTI (T9003), BBTI (T9777), azocasein, and other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).  
20   *E. coli* thioredoxin and NTR were isolated from cells transformed to overexpress each protein. The thioredoxin strain containing the recombinant plasmid, pFPI, was kindly provided by Dr. J.-P. Jacquot (de La Motte-Guery et al., 1991). The NTR strain containing the recombinant  
25   plasmid, pPMR21, was kindly provided by Drs. Marjorie Russel and Peter Model (Russel and Model, 1988). The isolation procedures used for these proteins were as described in those studies with the following changes: cells were broken in a Ribi cell fractionator at 25,000  
30   psi and NTR was purified as described by Florencio et al. (1988) without the red agarose step. The *E. coli* thioredoxin and NTR were, respectively, 100% and 90% pure as determined by SDS-polyacrylamide gel electrophoresis. Wheat thioredoxin h was purified as  
35   previously described (Johnson et al., 1987).

### Germination of Wheat Seeds

Wheat seeds were sterilized by steeping in 50% (v/v) of Generic Bleach for 1 h at room temperature, followed by a thorough wash with distilled water. The sterilized seeds were placed in a plastic Petri dish on two layers of Whatman filter paper moistened with distilled water containing 100  $\mu$ g/ml of chloramphenicol. Germination was continued at room temperature in a dark chamber for up to 5 days.

### 10 Preparation of Wheat Proteases

The endosperm (10-15 g fresh weight) isolated from 5-day germinated wheat seeds by excising the roots and shoots was extracted for 30 minutes at 4°C with 5 volumes of 200 mM sodium acetate, pH 4.6, containing 10 mM  $\beta$ -mercaptoethanol. The homogenate was centrifuged for 20 minutes at 48,000 g, 4°C. The pellet was discarded and the supernatant fluid was fractionated with 30-70% ammonium sulfate. This fraction, which represented the protease preparation, was resuspended in a minimum volume of 20 mM sodium acetate, pH 4.6, containing 10 mM  $\beta$ -mercaptoethanol, and dialyzed against this buffer overnight at 4°C. When assayed with azocasein as substrate, the protease preparation had an optimal pH of about 4.6 and was stable for at least one week at 4°C.

### Reduction and Proteolytic Susceptibility of Trypsin Inhibitors

Unless indicated, the reduction of the trypsin inhibitors (0.4 mg/ml) was carried out in 0.1 ml of 20 mM sodium phosphate buffer, pH 7.9 containing 10 mM EDTA at 30°C for 2 hours. The concentrations of thioredoxin, NTR, and NADPH were 0.024 mg/ml, 0.02 mg/ml, and 0.25 mM, respectively. With DTT as reductant, EDTA and components of the NADP/thioredoxin system were omitted. Following reduction, aliquots of the inhibitor mixture

were withdrawn either for determination of trypsin inhibitory activity or proteolytic susceptibility. In the subtilisin tests, the inhibitor mixture (50  $\mu$ l) was directly mixed with subtilisin and incubated at room temperature for 1 hour. With the wheat protease preparation, the pH of the inhibitor mixture (50  $\mu$ l) was first adjusted to 4.7 by mixing with 35  $\mu$ l of 200 mM sodium acetate, pH 4.6; 10  $\mu$ l of the wheat protease preparation was then added and incubation was continued for 2 hours at 37°C. To stop digestion with subtilisin, 2  $\mu$ l of 100 mM phenylmethylsulfonyl fluoride (PMSF) and 10  $\mu$ l of 10% SDS were added to the digestion mixture. With the plant protease preparation, digestion was stopped by adding an equal volume of SDS sample buffer [0.125 M Tris-HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v)  $\beta$ -mercaptoethanol, and 0.02% (w/v) bromophenol blue]. Proteolytic products were analyzed by electrophoresis with 12% or 16% SDS polyacrylamide slab gels (Laemmli, 1970). The dried slab gels were scanned with a laser densitometer (Pharmacia-LKB UltraScan XL) and the peak area of the KTI or BBTI protein band was obtained by integration with a Pharmacia GelScan XL software program.

#### Assays

Thioredoxin and NTR were assayed as previously described by Florencio et al. (1988). Trypsin activity was measured in 50 mM Tris-HCl, pH 7.9, by following the increase in absorbance at 253 nm with N-benzoyl-L-arginine ethyl ester as substrate (Mundy et al., 1984) or by the release of azo dye into the trichloroacetic acid (TCA)-soluble fraction from azocasein substrate (see below). For trypsin inhibition assays, trypsin (5 to 10  $\mu$ g) was preincubated with appropriate amounts of KTI or BBTI for 5 minutes at room temperature in 50 mM Tris-HCl, pH 7.9 and proteolytic activity was then determined. While the two substrates yielded

similar data, results are presented with only one substrate.

Wheat protease activity was measured by following the release of azo dye into TCA solution from azocasein substrate at pH 4.7. Fifty  $\mu$ l of wheat protease in a solution of 20 mM sodium acetate, pH 4.6, and 10 mM  $\beta$ -mercaptoethanol were added to 50  $\mu$ l of 200 mM sodium acetate, pH 4.6, and 100  $\mu$ l of 2% azocasein (in 20 mM sodium phosphate, pH 7.0). Following 1-hour incubation at 37°C, 1 ml of 10% TCA was added and the mixture was allowed to stand for 10 minutes at room temperature. After centrifugation for 5 minutes in a microfuge (8000 g), 1 ml of the supernatant solution was withdrawn and mixed with 1 ml of 1 N NaOH. The absorbance was read at 440 nm. Protein concentration was determined with Bio-Rad reagent using bovine serum albumin as a standard (Bradford, 1976).

## RESULTS

### Trypsin Inhibitory Activity

The 20 kDa Kunitz and 8 kDa Bowman-Birk trypsin inhibitors of soybean contain 2 and 7 disulfide groups, respectively (Birk, 1976; Wilson, 1988). Although their physiological functions have not been established, the two types of inhibitors have been extensively investigated owing to their wide distribution in legume seeds and their potential to cause nutritional disorders, e.g., hypertrophy and associated malfunctions of the pancreas. As shown in Tables I and II and described in previous Examples, KTI and BBTI are reduced specifically by the NADP/thioredoxin system from either *E. coli* or plants. The reduced forms of glutathione and glutaredoxin (a thiol protein capable of replacing thioredoxin in certain animal and bacterial systems, but not known to occur in plants (Holmgren, 1985)) were without effect.

To determine the consequence of reduction by thioredoxin, the trypsin inhibitory activity of the oxidized and reduced forms of KTI and BBTI was compared. As shown in Table XI, preincubation with the  
5 NADP/thioredoxin system (NTS) for 2 hours at 30°C resulted in a substantial loss of trypsin inhibitory activity (*i.e.*, there was an increase in trypsin activity relative to the uninhibited control). More specifically, the NADP/thioredoxin system effected a 3-  
10 and 6-fold increase in trypsin activity for KTI and BBTI, respectively. Similar results were obtained with DTT, a nonphysiological substitute for thioredoxin, and with thioredoxin reduced by lipoic acid, a naturally occurring dithiol. Extended incubation with DTT alone  
15 (overnight at room temperature) led to complete or almost complete inactivation of both inhibitors (data not shown). Unlike DTT, lipoic acid did not reduce (inactivate) KTI and BBTI significantly in the absence of thioredoxin.

TABLE XI

Changes in the Ability of Soybean Trypsin Inhibitors  
to Inhibit Trypsin Following Reduction by the  
NADP/Thioredoxin System, DTT or Reduced Lipoic Acid

| 5  | Treatment                        | Relative Trypsin Activity* |      |
|----|----------------------------------|----------------------------|------|
|    |                                  | KTI                        | BBTI |
|    | No inhibitor                     | 100                        | 100  |
|    | Inhibitor                        |                            |      |
|    | Oxidized                         | 17.0                       | 11.5 |
|    | Reduced by NTS <sup>1</sup>      | 55.6                       | 70.6 |
| 10 | Reduced by DTT <sup>2</sup>      | 68.6                       | 88.9 |
|    | Reduced by LA/Trx h <sup>3</sup> | 40.5                       | 87.8 |

\*The specific activity of the uninhibited control trypsin was 0.018  $\Delta A_{253nm}/\mu g/min$  using N-benzoyl-L-arginine ethyl ester as substrate.

15 <sup>1</sup>Reduction by *E. coli* NTS (NADP/thioredoxin system) was conducted at 30°C for 2 hours.

<sup>2</sup>Reduction by DTT (1 mM) was conducted at 30°C for 1 hour.

20 <sup>3</sup>Reduction by lipoic acid (LA, 0.4 mM) and wheat thioredoxin h (Trx h) was conducted at 30°C for 1 hour. In the presence of lipoic acid alone (0.4 mM), trypsin activity was 20.0% for KTI and 12.5% for BBTI.

Friedman and colleagues observed that heating soybean flour in the presence of sulfur reductants (sodium sulfite, N-acetyl-L-cysteine, reduced glutathione, or  
25 L-cysteine) inactivated trypsin inhibitors, presumably as a result of the reduction or interchange of disulfide groups with other proteins in soy flour (Friedman and Gumbmann, 1986; Friedman et al., 1982, 1984).  
30 Inactivation of the trypsin inhibitors by these reductants improved the digestibility and nutritive

value of flours in tested rats (Friedman and Gumbman, 1986). Taken together with these earlier observations, the present findings demonstrate that disulfide bonds of both KTI and BBTI targeted by thioredoxin are  
5 important to maintenance of trypsin inhibitory activity.

#### Heat Stability

Protease inhibitor proteins are typically stable to inactivation treatments such as heat. This stability is attributed, at least in part, to the cross-linking of  
10 disulfide bonds (Birk, 1976; Ryan, 1981). It is known that breaking the disulfide bonds by reduction decreases heat stability (Friedman et al., 1982). The question arises as to whether reduction by thioredoxin yields similar results.

15 The results as shown in TABLE XII provide a positive answer to this question. When heated at 80°C for 15 minutes, the thioredoxin-reduced form of KTI completely lost its ability to inhibit trypsin, whereas its oxidized counterpart retained about half of the original  
20 activity (Table XII). Oxidized BBTI was even more stable, retaining the bulk of its trypsin inhibitory activity after heating at 100°C for 25 minutes. Nonetheless, as with KTI, the reduced form of BBTI was fully inactivated by heat (Table XII). These results are  
25 consistent with prior observations (i) that KTI and BBTI show increased sensitivity to heat on reduction; and (ii) that pure BBTI in solution is more heat-stable than pure KTI in solution. The reverse is true for flour (i.e., KTI is more heat-stable than BBTI (Friedman et  
30 al., 1982 and 1991; and DiPietro and Liener, 1989)).

TABLE XII

Heat Stability of the Kunitz and Bowman-Birk  
Trypsin Inhibitors: Oxidized and Following Reduction  
by the *E. coli* NADP/thioredoxin System

| 5  | Treatment                            | Relative Trypsin Activity* |                 |
|----|--------------------------------------|----------------------------|-----------------|
|    |                                      | KTI                        | BBTI            |
|    | No inhibitor                         | 100                        | 100             |
|    | Inhibitor, unheated                  |                            |                 |
|    | Oxidized                             | 26.6                       | 9.4             |
|    | Reduced                              | 76.4                       | 82.4            |
| 10 | Inhibitor, heated 15 min<br>at 80°C  |                            |                 |
|    | Oxidized                             | 52.3                       | nd <sup>1</sup> |
|    | Reduced                              | 98.7                       | nd              |
| 15 | Inhibitor, heated 25 min<br>at 100°C |                            |                 |
|    | Oxidized                             | nd                         | 17.2            |
|    | Reduced                              | nd                         | 98.4            |

\*The specific activity of trypsin was 0.319  $\Delta A_{440\text{nm}}$ /mg/min using azocasein as substrate. The temperatures used for inactivation were determined in initial experiments designed to show the heat stability of the trypsin inhibitors under our conditions.

<sup>1</sup>nd: not determined.

#### Protease Susceptibility

To test whether the reduced forms of KTI and BBTI show decreased stability to proteases other than trypsin, both the reduced and oxidized forms of KTI and BBTI were incubated with a wheat protease preparation or with subtilisin and the proteolytic products were analyzed by SDS-PAGE. The extent of proteolysis was determined by measuring the abundance of intact protein on SDS gels

by laser densitometer. When tested with a protease preparation from 5-day germinated wheat seeds, the oxidized form of the Kunitz inhibitor was almost completely resistant to digestion whereas the

5   thioredoxin-reduced form was susceptible to protease. As shown in Table XIII, about 80% of KTI was degraded in a reaction that depended on all components of the NADP/thioredoxin system (NTS). BBTI showed the same pattern except that the oxidized protein showed greater

10   proteolytic susceptibility relative to KTI. Similar effects were observed with both inhibitors when the plant protease preparation was replaced by subtilisin (data not shown). The nature of the proteolytic reaction was not investigated, but it is noted that

15   peptide products were not detected on SDS gels.

TABLE XIII

Effect of Thioredoxin-linked Reduction  
on the Susceptibility of  
Kunitz and Bowman-Birk Trypsin Inhibitors  
to Proteolysis by a Plant Protease Preparation<sup>1</sup>

5

| Treatment                          | Relative Abundance <sup>2</sup> |                 |
|------------------------------------|---------------------------------|-----------------|
|                                    | KTI                             | BBTI            |
| No protease                        | 100                             | 100             |
| Protease                           |                                 |                 |
| No reduction system                | 97.9                            | 67.2            |
| 10 <i>E. coli</i> NTS <sup>3</sup> | 22.1                            | 16.0            |
| NTS minus thioredoxin              | 90.2                            | nd <sup>4</sup> |
| NTS minus NADPH                    | 97.7                            | nd              |
| NTS minus NTR                      | 97.9                            | nd              |

15 <sup>1</sup>Following reduction by *E. coli* thioredoxin system at 30°C for 2 hours, pH was adjusted to 4.7 by addition of 200 mM sodium acetate, pH 4.6. Wheat protease preparation was then added and incubated at 37°C for 2 hours, followed by SDS-PAGE analyses.

<sup>2</sup>Determined by laser densitometer.

20 <sup>3</sup>NTS: NADP/thioredoxin system.

<sup>4</sup>nd: not determined.

This Example shows that reduction by thioredoxin, or dithiothreitol (DTT), leads to inactivation of both proteins and to an increase in their heat and protease  
25 susceptibility. The results indicate that thioredoxin-linked reduction of the inhibitor proteins is relevant both to their industrial processing and to seed germination.

These results confirm the conclusion that disulfide bonds are essential for the trypsin inhibitory activity of KTI and BBTI (Birk, 1985; Friedman and Gumbmann, 1986; Friedman *et al.*, 1982, 1984). These studies also show that reduction (inactivation) can take place under physiological conditions (*i.e.*, at low temperature with NADPH-reduced thioredoxin). The ability to inactivate the trypsin inhibitors at lower temperatures provides a potential method for full inactivation of both trypsin inhibitors, thereby improving the quality of soybean products and saving energy. The need for a method for the complete inactivation of KTI is significant since 20% of its activity is consistently retained in soy flour under conditions in which BBTI is fully inactivated (Friedman *et al.*, 1991).

The present results also add new information on the protease susceptibility of KTI and BBTI. Their increase in protease susceptibility following reduction suggests that, if exposed to the protease inhibitors during seed germination, the NADP/thioredoxin system could serve as a mechanism by which the inhibitor proteins are modified (inactivated) and eventually degraded (Baumgartner and Chrispeels, 1976; Chrispeels and Baumgartner, 1978; Orf *et al.*, 1977; Wilson, 1988; Yoshikawa *et al.*, 1979). As stated previously, there is evidence that the NADP-thioredoxin system plays a similar role in mobilizing proteins during the germination of wheat seeds.

#### EXAMPLE 20

##### Reduction of Castor Seed 2S Albumin Protein by Thioredoxin

The results of the following study of sulfhydryl agents to reduce the 2S protein from castor seed (Sharief and Li, 1982; Youle and Huang, 1978) shows that thioredoxin

actively reduces intramolecular disulfides of the 2S large subunit but not the intermolecular disulfides joining the two subunits.

### Materials and Methods

#### 5 Materials

Seeds of castor (*Ricinus communis* L. var Hale) were obtained from Bothwell Enterprises, Plainview, TX). Biochemicals were obtained from Sigma Chemical Co. (St. Louis, MO). *E. coli* thioredoxin and NTR were isolated  
10 from cells transformed to overexpress each protein. The thioredoxin strain containing the recombinant plasmid pFPI, was kindly provided by Dr. J.-P. Jacquot (de La Mott-Guery et al. 1991). The strain containing the recombinant plasmid, pPMR21, was kindly provided by Drs.  
15 Marjorie Russel and Peter Model (Russel and Model, 1988). Thioredoxin and NTR were purified by the respective procedures of de La Mott-Guery et al. (1991) and Florencio et al. (1988). Reagents for SDS-polyacrylamide gel electrophoresis were purchased from  
20 Bio-Rad Laboratories (Richmond, CA). Monobromobimane (mBBBr) or Thiolite was obtained from Calbiochem (San Diego, CA). Other chemicals were obtained from commercial sources and were of the highest quality available. NADP-malate dehydrogenase and fructose-1,6-  
25 bisphosphatase were purified from leaves of corn (Jacquot et al. 1981) and spinach (Nishizawa et al. 1982), respectively. Thioredoxin h was isolated from wheat seeds by following the procedure devised for the spinach protein (Florencio et al. 1988). Glutathione  
30 reductase was prepared from spinach leaves (Florencio et al. 1988).

### Isolation of Protein Bodies

Protein bodies were isolated by a nonaqueous method (Yatsu and Jacks, 1968). Shelled dry castor seeds, 15 g, were blended with 40 ml of glycerol for 30 sec in a Waring blender. The mixture was filtered through four layers of nylon cloth. The crude extract was centrifuged at 272 x g for 5 min in a Beckman J2-21M centrifuge using a JS-20 rotor. After centrifugation, the supernatant fraction was collected and centrifuged 20 min at 41,400 x g. The pellet, containing the protein bodies, was resuspended in 10 ml glycerol and centrifuged as before (41,400 x g for 20 min) collecting the pellet. This washing step was repeated twice. The soluble ("matrix") fraction was obtained by extracting the pellet with 3 ml of 100 mM Tris-HCl buffer (pH 8.5). The remaining insoluble ("crystalloid") fraction, collected by centrifugation as before, was extracted with 3 ml of 6M urea in 100 mM Tris-HCl buffer (pH 8.5).

### 2S Protein Purification Procedure

The 2S protein was prepared by a modification of the method of Tully and Beevers (1976). The matrix protein fraction was applied to a DEAE-cellulose (DE-52) column equilibrated with 5 mM Tris-HCl buffer, pH 8.5 (Buffer A) and eluted with a 0 to 300 mM NaCl gradient in buffer A. Fractions containing the 2S protein were pooled and concentrated by freeze drying. The concentrated fraction was applied to a Pharmacia FPLC Superose-12 (HR 10/30) column equilibrated with buffer A containing 150 mM NaCl. The fraction containing 2S protein from the Superose-12 column was applied to an FPLC Mono Q HR 5/5 column equilibrated with buffer A. The column was eluted sequentially with 3 ml of buffer A, 20 ml of a linear gradient of 0 to 300 mM NaCl in buffer A and finally with buffer A containing 1 M NaCl. The 2S protein purified by this method was free of contaminants

in SDS polyacrylamide gels stained with Coomassie blue (Kobrehel et al., 1991).

#### Analytical Methods

Reduction of proteins was monitored by the  
5 monobromobimane (mBBBr)/SDS polyacrylamide gel electrophoresis procedure of Crawford et al. (1989). Labeled proteins were quantified as described previously in the "Reduction of Cereal Proteins, Materials and Methods" section. Protein was determined by the method  
10 of Bradford (1976).

#### Enzyme Assays/Reduction Experiments

The Wada et al., 1981 protocol was used for assaying NADP-malate dehydrogenase and fructose 1,6  
bisphosphatase in the presence of thioredoxin and 2S  
15 protein. Assays were conducted under conditions in which the amount of added thioredoxin was sufficient to reduce the castor 2S protein but insufficient to activate the target enzyme appreciably. All assays were at 25°C. Unless otherwise indicated, the thioredoxin  
20 and NTR used were from *E. coli*. The 2S protein was monitored during purification by mBBBr/SDS-polyacrylamide gel electrophoresis following its reduction by dithiothreitol and *E. coli* thioredoxin (Crawford et al., 1989; Kobrehel et al., 1991).

25 Fig. 39 represents the reduction of the matrix and crystalloid proteins from castor seed as determined by mBBBr/SDS-polyacrylamide gel electrophoresis procedure. 1 and 7, Control: no addition; 2 and 8, GSH/GR/NADPH: reduced glutathione, glutathione reductase (from spinach  
30 leaves) and NADPH; 3 and 9, NGS: NADPH, reduced glutathione, glutathione reductase (from spinach leaves) and glutaredoxin from *E. coli*; 4 and 10, NTS: NADPH, NTR, and thioredoxin (both proteins from *E. coli*); 5 and 11, NADPH; 6 and 12, NADPH and *E. coli* NTR. Reactions

- were carried out in 100 mM Tris-HCl buffer, pH 7.8. As indicated, 0.7  $\mu$ g NTR and 1  $\mu$ g thioredoxin were added to 70  $\mu$ l of this buffer containing 1 mM NADPH and target protein: 8  $\mu$ g matrix protein for treatments 1-6 and 10  $\mu$ g crystalloid protein for treatments 7-12. Assays with glutathione were performed similarly, but at a final concentration of 2 mM, 1.4  $\mu$ g glutathione reductase, 1  $\mu$ g glutaredoxin, and 1.5 mM NADPH. Reaction time was 20 min.
- 10 Fig. 40 represents the specificity of thioredoxin for reducing the disulfide bonds of castor seed 2S protein. (1) Control (no addition); (2) Control + NTS (same conditions as in Fig. 39); (3) Control (heated 3 min at 100°C); (4) Control + 2 mM DTT (heated 3 min at 100°C).
- 15 The samples containing 5  $\mu$ g 2S protein and the indicated additions were incubated for 20 min in 30 mM Tris-HCl (pH 7.8). mBBBr, 80 nmol, was then added and the reaction continued for another 15 min prior to analysis by the mBBBr/SDS polyacrylamide gel electrophoresis
- 20 procedure.

### Results

- The castor storage proteins, which are retained within a protein body during seed maturation, can be separated into two fractions on the basis of their solubility. The more soluble proteins are housed in the protein body outer section ("matrix") and the less soluble in the inner ("crystalloid"). In the current study, the matrix and crystalloid components were isolated to determine their ability to undergo reduction by cellular thiols, viz., glutathione, glutaredoxin and thioredoxin. Glutaredoxin, a 12 kDa protein with a catalytically active thiol group, can replace thioredoxin in certain enzymic reactions of bacteria and animals (Holmgren et al. 1985) but is not known to occur in plants.

Fig. 39 shows that, while a number of storage proteins of castor seed were reduced by the thiols tested, only a low molecular weight protein, corresponding to the large subunit of the 2S protein of the matrix, showed strict specificity for thioredoxin. Certain higher molecular weight proteins of the crystalloid fraction underwent reduction, but in those cases there was little difference between glutaredoxin and thioredoxin (Fig. 39). The castor seed 2S large subunit thus appeared to resemble cystine-containing proteins previously discussed in undergoing thioredoxin-specific reduction. These experiments were designed to confirm this specificity and to elucidate certain properties of the reduced protein. As expected, owing to lack of disulfide groups, the 2S small subunit showed essentially no reaction with mBBBr with any of the reductants tested.

When its fluorescent band was monitored by laser densitometry, the reduction of the castor seed 2S large subunit was found to depend on all components of the NADP/thioredoxin system (NADPH, NTR and thioredoxin) (Table XIV). As for other thioredoxin-linked proteins (including chloroplast enzymes), the thioredoxin active in reduction of the 2S large subunit could be reduced either chemically with dithiothreitol (DTT) or enzymatically with NADPH and NTR. The extent of reduction by the NADP thioredoxin system, DTT alone, and DTT + thioredoxin was 84%, 67% and 90%, respectively, after 20 min at 25°C. Similar, though generally extensive reduction was observed with the disulfide proteins discussed above (Johnson et al. 1987). As with the other seed proteins, native wheat thioredoxin *h* and *E. coli* thioredoxins could be used interchangeably in the reduction of the 2S protein by DTT (data not shown).

TABLE XIV

Extent of reduction of the castor seed 2S protein by different sulfhydryl reductants. Reaction conditions as in Fig. 39. A reduction of 100% corresponds to that obtained when the 2S protein was heated for 3 min in the presence of 2% SDS and 2.5%  $\beta$ -mercaptoethanol. NTS: NADPH, NTR, and thioredoxin (both proteins from *E. coli*); GSH/GR/NADPH: reduced glutathione, glutathione reductase (from spinach leaves) and NADPH; NGS: NADPH, reduced glutathione, glutathione reductase (from spinach leaves) and glutaredoxin (from *E. coli*).

| <u>Treatment</u>                   | <u>Relative Reduction, %</u> |
|------------------------------------|------------------------------|
| Control                            | 0                            |
| NADP/thioredoxin system, complete  | 84                           |
| 15     "     minus thioredoxin     | 0                            |
| "     minus NADPH                  | 0                            |
| "     minus NTR                    | 0                            |
| Reduced glutathione                | 0                            |
| NADP/glutaredoxin system, complete | 0                            |
| 20     DTT                         | 67                           |
| DTT + Thioredoxin                  | 90                           |

The capability of thioredoxin to reduce the castor seed 2S protein was also evident in enzyme activation assays. Here, the protein targeted by thioredoxin (in this case 2S) is used to activate a thioredoxin-linked enzyme of chloroplasts, NADP-malate dehydrogenase or fructose 1,6-bisphosphatase. As with most of the proteins examined so far, the 2S protein more effectively activated NADP-malate dehydrogenase and showed little activity with the fructose bisphosphatase (2.6 vs. 0.0 nmoles/min/mg protein).

The castor seed 2S protein contains inter-as well as intramolecular disulfides. The 2S protein thus provides an opportunity to determine the specificity of thioredoxin for these two types of bonds. To this end, 5 the castor seed 2S protein was reduced (i) enzymically with the NADP/thioredoxin system at room temperature, and (ii) chemically with DTT at 100°C. Following reaction with mBBR the reduced proteins were analyzed by SDS-polyacrylamide gel electrophoresis carried out 10 without additional sulfhydryl agent. The results (Fig. 40) indicate that while thioredoxin actively reduced intramolecular disulfides, it was much less effective with intermolecular disulfides.

The present results extend the role of thioredoxin to 15 the reduction of the 2S protein of castor seed, an oil producing plant. Thioredoxin specifically reduced the intramolecular disulfides of the large subunit of the 2S protein and showed little activity for the intermolecular disulfides joining the large and small 20 subunits. Based on the results with the trypsin inhibitors of soybean, it is clear that reduction of intramolecular disulfides by thioredoxin markedly increases the susceptibility of disulfide proteins to proteolysis (Jiao et al. 1992a). It, however, remains 25 to be seen whether reduction of the 2S protein takes place prior to its proteolytic degradation (Youle and Huang, 1978) as appears to be the case for the major storage proteins of wheat. A related question raised by this work is whether the 2S protein of castor, as 30 well as other oil producing plants such as brazil nut (Altenbach et al., 1987; Ampe et al., 1986), has a function in addition to that of a storage protein.

EXAMPLE 21

Thioredoxin-Dependent Deinhibition  
of Pullulanase of Cereals by  
Inactivation of a Specific Inhibitor Protein

5    Assay of Pullulanase

## 1.    Standard curve of maltotriose:

A series of concentrations of maltotriose (0 to 2 mg) in 0.1 to 0.2 ml water or buffer were made in microfuge tubes. To this was added 0.2 ml of dinitrosalicylic acid (DA) reagent (mix 1 g of DA, 30 g of sodium potassium tartrate, and 20 ml of 2N NaOH with water to final volume of 100 ml). The reagents were dissolved in a warm water bath. The mixture was heated at 100°C for 5 min and cooled down in a water bath (room temperature). Each sample was transferred to a glass tube that contained 2 ml of water. Read  $A_{493}$  vs water.  $\Delta A_{493}$  [ $A_{493}$  of sample containing maltotriose was subtracted from  $A_{493}$  of the blank (no maltotriose)] was plotted against maltotriose concentrations.

## 20    2.    Pullulanase activity assay

Pullulanase activity is measured as the release of reducing sugar from the substrate pullulan. Typically 10-100  $\mu$ l of pullulanase sample (in 20 mM Tris-HCl, pH 7.5, or in 5-20 acetate-NA, pH 4.6) was mixed with 25-100  $\mu$ l of 200 mM Acetate-NA, pH 5.5 (this buffer serves to bring final pH of the assay to 5.5) and 10-20  $\mu$ l of 2% (w/v) pullulan. The mixture was incubated at 37°C for 30 to 120 min, depending on the activity of pullulanase. The reaction was stopped by adding 200  $\mu$ l of DA reagent. Reducing sugar was then determined as above.

## Note:

1.    When a crude extract of pullulanase obtained by the dialysis of crude extracts or pullulanase

obtained from a dialyzed 30-60% ammonium sulfate fraction is used as a pullulanase source, it must be thoroughly dialysed before assay because there are reduced sugars in the crude extract. In other words the background of crude pullulanase samples from dialysed crude extracts or a dialysed 30-60% ammonium sulfate fraction is very high. In this case, the blank is made as follows: 200  $\mu$ l of DA reagent are added first, followed by the addition of enzyme sample, pullulan and buffer.

2. When final concentrations of DTT (or  $\beta$ -mercaptoethanol (MET) or GSH) are higher than 2 mM in the assay mixture, the OD<sub>493</sub> values will be greater than those of the minus-DTT (MET, GSH) samples. DTT (MET, GSH) should be added to the blank, samples without DTT during assay at the end of the reaction. Care should be taken to make sure the final concentration of DTT in the assay mixture is below 2 mM.

#### Purification of Pullulanase Inhibitor Extraction and Ammonium Sulfate Fractionation

200 g of barley malt was ground to fine powder with an electric coffee grinder and extracted with 600 ml of 5% (w/v) NaCl for 3 h at 30°C. Following centrifugation at 30,000 g and at 4°C for 25 min, the supernatant was fractionated by precipitation with solid ammonium sulfate. Proteins precipitated between 30% and 60% saturated ammonium sulfate were dissolved in a minimum volume of 20 mM Tris HCl, pH 7.5, and dialyzed against this buffer at 4°C overnight.

#### DE52 Chromatography

The dialyzed sample was centrifuged to remove insoluble materials and the supernatant adjusted to pH 4.6 with 2N formic acid. After pelleting the acid-denatured protein, the supernatant was readjusted to pH 7.5 with NH<sub>4</sub>OH and loaded onto a DE52 column (2.5 x 26 cm)

equilibrated with 20 mM Tris-HCl, pH 7.5. Following wash with 80 ml of the above buffer, the column was eluted with a linear 0-500 mM Tris-HCl, pH 7.5. Fractions of 6.7 ml were collected. Pullulanase was  
5 eluted at about 325 mM NaCl and its inhibitor came off at about 100 mM NaCl. Pullulanase was further purified through CM32 (20 mM sodium acetate, pH 4.6) and Sephacryl-200 HR (30 mM Tris-HCl, pH 7.5, containing 200 mM NaCl and 1 mM EDTA) chromatography. Pullulanase  
10 inhibitor protein was purified as described below.

#### CM32 Chromatography

The pullulanase inhibitor sample (about 90 ml) from the DE52 step was placed in a 150-ml flask and incubated at 70°C water-bath for 20 min. Following centrifugation,  
15 the clarified sample was then adjusted to pH 4.6 with 2N formic acid and dialyzed against 20 mM sodium acetate, pH 4.6. The precipitate formed during dialysis was removed by centrifugation and the supernatant was chromatographed on a CM32 column (2.5 X 6 cm)  
20 equilibrated with 20 mM sodium acetate, pH 4.6. Proteins were eluted with a linear 0-0.4 M NaCl in 200 ml of 20 mM sodium acetate, pH 4.6. Fractions (5.0 ml/fraction) containing pullulanase inhibitory activity were pooled, dialyzed, and rechromatographed on a CM32  
25 column (2.5 x 6 cm) with a linear 0.2-1 M NaCl gradient in 200 ml of 20 mM sodium acetate, pH 4.0.

#### Sephadex G-75 Filtration

Pullulanase inhibitor fractions from the second CM32 chromatography step were concentrated in a dialysis bag  
30 against solid sucrose and then separated by a Sephadex G-75 column (2.5 x 85 cm) equilibrated with 30 mM Tris-HCl, pH 7.5, containing 200 mM Na Cl and 1 mM EDTA. Fractions (3.6 ml/fraction) showing pullulanase inhibitory activity were pooled, concentrated by

dialysis against solid sucrose, and then dialysed against 10 mM Tris-HCl, pH 7.5.

Identification and Purification  
of Pullulanase Inhibitor

- 5 During germination, starch is converted to glucose by  $\alpha$ -,  
 $\beta$ -amylases, and pullulanase (also called debranching  
enzyme, R-enzyme). While extensive studies have been  
conducted for the regulation of amylases, little is  
known about the regulation of pullulanase in seeds.
- 10 Yamada (Yamada, J. (1981) Carbohydrate Research 90:153-  
157) reported that incubation of cereal flours with  
reductants (e.g., DTT) or proteases (e.g., trypsin) led  
to an activation of pullulanase activity, suggesting  
that reduction or proteolysis might be a mechanism by  
15 which pullulanase is activated during germination. Like  
in rice flour, pullulanase extracts from germinated  
wheat seeds or from barley malt showed lower activity,  
and were activated 3 to 5-fold by preincubation with DTT  
for 20 to 30 min. However, following purification of  
20 the crude extract (a dialysate of 30-60% ammonium  
sulfate fraction) by anion or cation exchange  
chromatography, the total pullulanase activity increased  
2 to 3-fold over that of the sample applied to the  
column when assayed without preincubation with DTT, and  
25 DTT has no or little effect on pullulanase. One  
possibility was that pullulanase might be activated by  
proteolysis during the process of purification, since  
germinated wheat seeds or barley malt show high protease  
activity. If this was the case, addition of protease  
30 inhibitor cocktail would prevent pullulanase activation  
during purification. In contrast to this point, many  
experiments with protease inhibitors failed to prove  
this. Another possibility was that there is an  
inhibitor that is precipitated by ammonium sulfate and  
35 inhibits pullulanase. The role of DTT is to reduce and  
thus inactivate this protein inhibitor, leading to

- activation of pullulanase. Along this line, the 30-60% ammonium sulfate fraction from barley malt was applied to a DE52 column (2.5 x 26 cm) equilibrated with 20 mM Tris-CH<sub>1</sub>, pH 7.5 (Fig. 41). Following elution with a linear salt gradient, "deinhibited" ("activated") pullulanase was identified as a protein peak coming off at about 325 mM NaCl (from fraction numbers 44 to 60). Assay of pullulanase activity in the preincubation mixture consisting of 50  $\mu$ l of the peak pullulanase activity fraction (fraction number 45) with 50  $\mu$ l of other protein fractions indicated that a protein peak that showed pullulanase inhibitory activity was eluted from the DE52 column by about 100 mM NaCl between fraction numbers 8 to 25 (Fig. 41).
- 15 The pullulanase inhibitor sample was further purified by two consecutive cation exchange chromatography steps with CM32 at pH 4.6 (Fig. 42) and 4.0 (Fig. 43) and filtration with Sephadex G-75 (Fig. 44).

#### Properties of Pullulanase Inhibitor

- 20 Preliminary experiments showed that pullulanase inhibitor protein is resistant to treatment of 70°C for 10 min and pH 4.0. Based on the profile of Sephadex G-75 gel filtration and SDS-PAGE, pullulanase inhibitor has a molecular weight between 8 to 15 kDa  $\pm$  2 kDa. The study further showed that the protein contains thioresoxin-reducible (S-S) bonds.

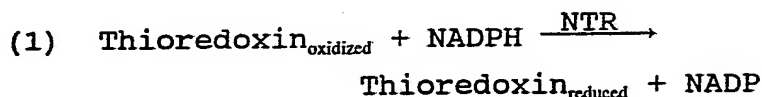
- These studies, as shown in Table XV, found that the ubiquitous dithiol protein, thioresoxin, serves as a specific reductant for a previously unknown disulfide-containing protein that inhibits pullulanase of barley and wheat endosperm.

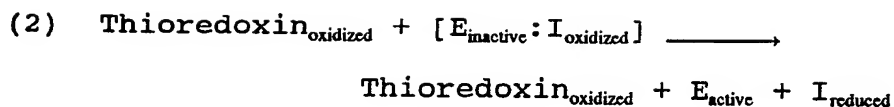
TABLE XV

Activity Change in Pullulanase Inhibitor Protein  
Following Reduction by NADP/Thioredoxin System

| 5  | Treatment                         | Relative<br>Pullulanase<br>Activity |
|----|-----------------------------------|-------------------------------------|
|    | No inhibitor                      | 100                                 |
|    | Inhibitor                         |                                     |
|    | Oxidized                          | 30.1                                |
| 10 | Reduced by DTT                    | 46.1                                |
|    | Reduced by <i>E. coli</i> Trx/DTT | 95.1                                |
|    | Reduced by <i>E. coli</i> NTS     | 40.4                                |
|    | Reduced by GSH/NADPH/GR           | 33.6                                |

Reduction of the inhibitor protein eliminated its  
15 ability to inhibit pullulanase, thereby rendering the  
pullulanase enzyme active. These studies as shown in  
Table XV illustrate that it is possible to render the  
pullulanase enzyme active with a physiological system  
consisting of NADPH, NADP-thioredoxin reductase (NTR)  
20 and thioredoxin (the NADP/thioredoxin system) as well  
as with thioredoxin (Trx) and dithiothreitol. These  
findings also elucidate how reductive activation of  
pullulanase takes place (*i.e.*, that a specific  
(previously unknown) inhibitor is reduced and thereby  
25 inactivated, so that the enzyme becomes active). The  
thioredoxin active in this reaction can be obtained from  
several sources such as *E. coli* or seed endosperm  
(thioredoxin *h*). The role of thioredoxin in reductively  
inactivating the inhibitor protein (I) and deinhibiting  
30 the pullulanase enzyme (E) is given in Equations 1  
and 2.





In summary, the crude endosperm extracts were fractionated by column chromatography procedures. These steps served to separate the protein inhibitor from the pululanase enzyme. The inhibitor protein was then highly purified by several steps. By use of the mBBR/SDS-PAGE procedure, it was determined that disulfide group(s) of the new protein are specifically reduced by thioredoxin and that the reduced protein loses its ability to inhibit pullulanase. Like certain other disulfide proteins of seeds (e.g., the Kunitz and Bowman-Birk trypsin inhibitors of soybean), the pullulanase inhibitor protein showed the capability to activate chloroplast NADP-malate dehydrogenase. In these experiments, dithiothreitol was used to reduce thioredoxin, which in turn reduced inhibitor and thereby activated the dehydrogenase enzyme.

#### EXAMPLE 22

#### 20      Engineering of Yeast Cells to Overexpress Thioredoxin and NADP-Thioredoxin Reductase

The two *Saccharomyces cerevisiae* thioredoxin genes (Muller, E.G.D. (1991), J. Biol. Chem. 266:9194-9202), TRX1 and TRX2, are cloned in high copy number episomal vectors, an example of which is YEp24, under the control of strong constitutive promoter elements, examples of which are the glycolytic promoters for the glyceraldehyde-3-P dehydrogenase, enolase, or phosphoglycerate kinase genes. Recombinant constructs are assessed for the overexpression of thioredoxin by quantitative Western blotting methods using an antithioredoxin rabbit antiserum (Muller, E.G.D., et al. (1989), J. Biol. Chem. 264:4008-4014), to select the

optimal combination of thioredoxin genes and promoter elements. The cells with the optimal thioredoxin overexpression system are used as a source of thioredoxin for dough improvement.

- 5 The NADP-thioredoxin reductase gene is cloned by preparing an oligonucleotide probe deduced from its amino terminal sequence. The enzyme is prepared from yeast cells by following a modification of the procedure devised for spinach leaves (Florencio, F.J., et al.
- 10 (1988), Arch. Biochem. Biophys. 266:496-507). The amino terminus of the pure reductase enzyme is determined by microsequencing by automated Exman degradation with an Applied Biosystems gas-phase protein sequencer. On the basis of this sequence, and relying on codon usage in
- 15 yeast, a 20-base 24-fold degenerate DNA probe is prepared. The probe is hybridized to isolated yeast DNA cleaved with EcoRI and PstI by Southern blot analysis. The most actively region is extracted from the agarose gels and introduced into a pUC19 plasmid vector
- 20 (Szekeres, M., et al. (1991), J. Bacteriol. 173:1821-1823). Following transformation, plasmid-containing *E. coli* colonies are screened by colony hybridization using the labeled oligonucleotide probe (Vogeli, G., et al. (1987), Methods Enzymol. 152:407-415). The clone is
- 25 identified as carrying the gene for NADP-thioredoxin reductase by sequencing the DNA as given in Szekeres, et al. above. Once identified, the NADP-thioredoxin reductase gene is overexpressed in yeast as described above for the TRX1 and TRX2 yeast thioredoxin genes.
- 30 The yeast cells which overexpress NADP-thioredoxin reductase are used as a source of reductase to improve dough quality.

EXAMPLE 23Improvement in Dough Quality  
Using Genetically Engineered Yeast Cells

*Saccharomyces cerevisiae* cells engineered to overexpress  
5 the two yeast thioredoxins and the yeast NADP-  
thioredoxin reductase as set forth in Example 23 are  
lysed by an established procedure such as sonication and  
then freeze dried. The dried cells from the cultures  
overexpressing thioredoxin and NADP-thioredoxin  
10 reductase are combined and then used to supplement flour  
to improve its dough quality. Two-tenths gram of the  
combined lysed dried cells are added together with about  
300 to about 500 nanomoles NADPH to 1 M Tris-HCl buffer,  
pH 7.9, to give 5.25 ml of 30 mM Tris-HCl. The reaction  
15 is carried out in a microfarinograph at 30°C as  
described in Example 14. An improvement in dough  
quality is observed which is similar to the improvement  
shown in Example 14.

EXAMPLE 24Improvement of Gluten

The positive effects of the NADP/thioredoxin system on  
dough quality presents the option of applying this  
system to flour in the preparation of gluten. The  
purpose is to alter the yield and the properties of  
25 gluten, thereby enhancing its technological value: (1)  
by obtaining stronger glutens (increased elasticity,  
improved extensibility); (2) by increasing gluten yield  
by capturing soluble proteins, reduced by the NADP-  
thioredoxin system, in the protein network, thereby  
30 preventing them from being washed out during the  
production of gluten. In this procedure (using 10 g  
flour), 0.2 µg *E. coli* thioredoxin, 0.1 µg *E. coli* NADP-  
thioredoxin reductase and 300 to 500 nanomoles NADPH are

added together with 1 M Tris-HCl, pH 7.9, buffer to give 5.25 ml of 30 mM Tris-HCl. The gluten is made at room temperature according to the common lixiviation method. The yield of the gluten is determined by weight and the strength of the gluten is determined by the classical manual stretch method. The gluten product which are obtained by this treatment with the NADP/thioredoxin system is used as an additive in flour or other grain.

#### EXAMPLE 25

##### 10                    Method of Producing Dough                       from a Non-wheat or Rye Flour

For this test (using 10 gm of milled flour from corn, rice or sorghum), 0.2  $\mu$ g *E. coli* thioredoxin, 0.1  $\mu$ g *E. coli* NADP-thioredoxin reductase and 500 nanomoles NADPH are added together with 1 M Tris-HCl, pH 7.9, buffer to give 5.25 ml of 30 mM Tris-HCl. The reaction is carried out by mixing the 10 gm of milled flour with the enzyme system in a micro-farinograph at 30°C. The farinograph measurements show wheat-like dough characteristics by the added NADP-thioredoxin system. In the controls without the enzyme system, no microfarinograph reading is possible because the mixture fails to form a dough. The dough which is formed is persistent and its consistency is maintained throughout the run. The end product is similar to the network formed in dough derived from wheat.

#### REDUCTION OF ANIMAL TOXINS

The invention provides a method for chemically reducing toxicity causing proteins contained in bee, scorpion and snake venome and thereby altering the biological activity of the venoms well as reducing the toxicity of

animal toxins specifically snake neurotoxins by means of thiol redox (SH) agents namely a reduced thioredoxin, reduced lipoic acid in the presence of a thioredoxin or DTT. The reduction of the thioredoxin occurs  
5       preferably via the NADP-thioredoxin system (NTS). As stated previously, the NTS comprises NADPH, NADP-thioredoxin reductase (NTR) and a thioredoxin.

The term "thiol Redox agent" has been used sometimes in the literature to denote both an agent in the nonreduced  
10       state and also in the reduced or sulfhydryl (SH) state. As defined herein the term "thiol redox (SH) agent" means a reduced thiol redox protein or synthetically prepared agent such as DTT.

The reduction of the neurotoxin may take place in a  
15       medium that is liquid such as blood, lymph or a buffer, etc. or in a medium that is solid such as cells or other living tissue. As used herein the term "liquid" by itself does not refer to a biological fluid present in an individual.

20       Presumably the proficiency of the thiol redox (SH) agents to inactivate the venom *in vitro* and to detoxify the venom in individuals depends upon the ability of the agents of the invention to reduce the intramolecular disulfide bonds in these toxicity causing venom  
25       components.

All snake neurotoxins, both presynaptic and postsynaptic can be reduced and at least partially inactivated *in vitro* by the thiol redox (SH) agents of the invention. Snake neurotoxins inactivated *in vitro* according to the  
30       invention are useful as antigens in the preparation of antivenoms. The neurotoxins are inactivated preferably by incubation with a thiol redox (SH) agent in an appropriate buffer. The preferred buffer is Tris-HCl

buffer but other buffers such as phosphate buffer may be used. The preferred thiol redox (SH) agent is a reduced thioredoxin.

Effective amounts for inactivating snake neurotoxins range from about 0.1  $\mu\text{g}$  to 5.0  $\mu\text{g}$ , preferably about 0.5  $\mu\text{g}$  to 1.0  $\mu\text{g}$ , of a reduced thioredoxin; from about 1 nanomole to 20 nanomoles, preferably from 5 nanomoles to 15 nanomoles, of reduced lipoic acid in the presence of about 1.0  $\mu\text{g}$  of a thioredoxin and from about 10 nanomoles to 200 nanomoles, preferably 50 nanomoles to 100 nanomoles, of reduced DTT (preferably in the presence of about 1.0  $\mu\text{g}$  of a thioredoxin) for every 10  $\mu\text{g}$  of snake neurotoxin in a volume of 100  $\mu\text{l}$ .

The effective amounts for inactivating a snake neurotoxin using the components in the NADP-thioredoxin system range from about 0.1  $\mu\text{g}$  to 5.0  $\mu\text{g}$ , preferably about 0.5  $\mu\text{g}$  to 1.0  $\mu\text{g}$ , of thioredoxin; from about 0.1  $\mu\text{g}$  to 2.0  $\mu\text{g}$ , preferably from 0.2  $\mu\text{g}$  to 1.0  $\mu\text{g}$ , of NTR and from about 0.05 micromoles to 0.5 micromoles, preferably about 0.1 micromoles to 0.25 micromoles, of NADPH for every 10  $\mu\text{g}$  of snake neurotoxin in a volume of 100  $\mu\text{l}$ .

Upon inactivation the buffer containing the inactivated neurotoxin and thiol redox (SH) agent, etc. may be injected into an animal such as a horse to produce an antivenom or prior to injection it may be further treated with heat or formaldehyde.

The thiol redox (SH) agents of the invention may also be used to treat individuals who are suffering the effects of neurotoxicity caused by a venomous snake bite. The preferred method of administering the reduced thiol redox (SH) agent to the individual is by multiple subcutaneous injections around the snake bite.

Of course the correct amount of a thiol redox (SH) agent used to detoxify a neurotoxin in an individual will depend upon the amount of toxin the individual actually received from the bite. However, effective amounts for detoxifying or reducing the toxicity of snake neurotoxins in mice usually range from about 0.01  $\mu\text{g}$  to 0.3  $\mu\text{g}$ , preferably about 0.02  $\mu\text{g}$  to 0.05  $\mu\text{g}$ , of a reduced thioredoxin; from about 0.1 nanomole to 3.0 nanomoles, preferably from 0.2 nanomole to 1.0 nanomole, of reduced lipoic acid in the presence of about 0.05  $\mu\text{g}$  of a thioredoxin; from about 1.0 nanomole to 30 nanomoles, preferably from 2.0 nanomoles to 5.0 nanomoles, of DTT, preferably in the presence of 0.05  $\mu\text{g}$  of a thioredoxin, for every gm of mouse body weight.

The effective amounts for detoxifying a snake neurotoxin in a mouse using the components of the NADP-thioredoxin system range from about 0.01  $\mu\text{g}$  to 0.3  $\mu\text{g}$ , preferably about 0.02  $\mu\text{g}$  to 0.05  $\mu\text{g}$  of a thioredoxin; from about 0.005  $\mu\text{g}$  to 0.12  $\mu\text{g}$ , preferably from 0.01  $\mu\text{g}$  to 0.025  $\mu\text{g}$ , of NTR and from about 5 nanomoles to 30 nanomoles, preferably 10 nanomoles to 15 nanomoles, NADPH for every gm of mouse body weight.

The preferred method of administering the NTS to an individual is also by multiple subcutaneous injections. The preferred thiol redox agent for human use is human thioredoxin administered via the NADP-thioredoxin system or with lipoic acid or DTT.

A partial list of the venomous snakes which produce the neurotoxins which can be inactivated or detoxified by the methods of this invention appears on pages 529-555 of Chippaur, J.-P., et al. (1991) Reptile Venoms and Toxins, A.T. Tu, ed., Marcel Dekker, Inc., which is herein incorporated by reference.

Other features and advantages of the invention with respect to inactivating and detoxifying venome can be ascertained from the following examples.

#### EXAMPLE 26

5            Reduction Studies of Bee, Scorpion  
             and Snake Venoms and Labeling with mBBr

Reactions were carried out with 50  $\mu$ g venom (final volume of 100  $\mu$ l) in 30 mM Tris-CHl buffer pH 7.9 containing the following protease inhibitors:  
10 phenylmethylsulfonyl fluoride (PMSF), leupeptin and pepstatin (final concentrations used in the assay respectively: 100  $\mu$ M, 1  $\mu$ M and 1  $\mu$ M). With NADPH as a reductant, the mixture also contained 4  $\mu$ g thioredoxin, 3.5  $\mu$ g NTR (both from *E. coli*) and 12.5 mM NADPH. When  
15 thioredoxin (4  $\mu$ g, *E. coli* or human) was reduced by DTT, NADPH and NTR were omitted and DTT was added to 0.5 mM. Assays with GSH were performed similarly but at a final concentration of 5 mM and in the presence of 1.5  $\mu$ g glutathione reductase and 12.5 mM NADPH. The mixture  
20 was incubated for 20 min at room temperature, mBBr was then added to 1.5 mM and the reaction was continued for 15 min at room temperature. The reaction was stopped and excess mBBr derivitized by adding 10  $\mu$ l of 100 mM  $\beta$ -mercaptoethanol, 5  $\mu$ l of 20% SDS and 10  $\mu$ l of 50%  
25 glycerol. Samples were then analyzed by SDS-polyacrylamide gel electrophoresis as previously described.

The same experiment with the NADP-thioredoxin system was performed without adding protease inhibitors.

30 The extent of the reduction of the bee, scorpion and snake venoms by different reductants described above is shown in Figs. 45, 46 and 47, respectively. Figs. 45,

46 and 47 represent the results of the reduction studies of different venoms (SDS-Polyacrylamide gel/mBBBr procedure). After 20 min incubation at room temperature with different reductants and in the presence of protease inhibitors, the samples were derivatized with mBBBr and separated by electrophoresis and fluorescence was determined. (Fig. 45: Bee venom from *Apis mellifera*; Fig. 46: scorpion venom from *Androctonus australis*, and Fig. 47: snake venom from *Bungarus multicinctus*). It may be seen that in all these cases thioredoxin (*E. coli* or human) specifically reduced components of the venoms. Fig. 48 shows that thioredoxin reduces venom components in a similar way when the reaction was performed in the absence of protease inhibitors.

Fig. 48 represents the results of the reduction of bee, scorpion and snake venoms by the NADP-Thioredoxin system with and without protease inhibitors (SDS-Polyacrylamide gel mBBBr procedure). After 20 min incubation at room temperature with NTS in the presence or absence of any protease inhibitors, the samples were derivatized with mBBBr, separated by electrophoresis, and fluorescence was determined as in Figs. 45-47.

#### Materials

25 Venoms: Bee venom from *Apis mellifera*, scorpion venom from *Androctonus australis*, and snake venom from *Bungarus multicinctus* were purchased from Sigma chemical Co. (St. Louis, MO).

Protease Inhibitors: Phenylmethylsulfonyl fluoride (PMSF), Leupeptin and Pepstatin were purchased from 30 Sigmal Chemical Co. (St. Louis, MO).

### Venom Detoxification

Detoxification of bee, scorpion and snake venoms is determined by subcutaneous injection into mice. Assays are done in triplicate. Prior to injection, the venom is diluted in phosphate-saline buffer (0.15 M NaCl in 10 mM  $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$  pH 7.2) at concentrations ranging up to twice the  $\text{LD}_{50}$  (per g mouse): bee venom from *Apis mellifera*, 2.8  $\mu\text{g}$ ; scorpion venom from *Androctonus australis*, 0.32  $\mu\text{g}$ ; and snake venom from *Bungarus multicinctus*, 0.16  $\mu\text{g}$ . At 5, 10, 30, 60 minutes and 4, 12 and 24 hr after injection, separate groups of challenged mice are injected (1) intravenously and (2) subcutaneously (multiple local injections around the initial injection site). The thioredoxin is reduced with : (1) *E. coli* NADP-thioredoxin system, using 0.08  $\mu\text{g}$  thioredoxin, 0.07  $\mu\text{g}$  NTR and 25 nmoles NADPH; (2) Thioredoxin reduced by DTT or reduced lipoic acid (0.08  $\mu\text{g}$  *E. coli* or human thioredoxin added to 1 nmole dithiothreitol or 1 nmole of reduced lipoic acid). Concentrations are per  $\mu\text{g}$  venom injected into the animal; all solutions are prepared in phosphate-saline buffer.

The effect of thioredoxin on detoxification is determined by (1) comparing the  $\text{LD}_{50}$  with the control group without thioredoxin and (2) following the extent of the local reaction, as evidenced by necrosis, swelling and general discomfort to the animal.

### REDUCTION STUDIES FOR REDUCING SNAKE NEUROTOXINS - MATERIALS AND METHODS

#### 30 Toxins

Porcine pancreas phospholipase  $\text{A}_2$ , erabutoxin b and  $\beta$ -bungarotoxin were purchased from Sigma Chemical Co. (St. Louis, Mo). As the phospholipase  $\text{A}_2$  was provided in 3.2 M  $(\text{NH}_4)_2\text{SO}_4$  solution pH 5.5, the protein was dialysed in

30 mM Tris-HCl buffer, pH 7.9, using a centricon 3 KDa cutoff membrane.  $\alpha$ -Bungarotoxin and  $\alpha$ -bungarotoxin<sup>125</sup>I were a kind gift from Dr. Shalla Verrall.

#### Reagents and Fine chemicals

- 5 DL- $\alpha$ -Lipoic acid, L- $\alpha$ -phosphatidylcholine from soybean, NADPH and  $\beta$ -mercaptoethanol were purchased from Sigma Chemical Co. (St Louis, Mo.) and monobromobimane (mBBR, trade name thiolite) from Calbiochem (San Diego, Ca.). Reagents for sodium dodecylsulfate (SDS)-polyacrylamide  
10 gel electrophoresis were purchased from Bio-Rad Laboratories (Richmond, Ca.).

#### Proteins and Enzymes

- Thioredoxin and NTR were purified from *E. coli* as is described by Jiao, et al., (1992) Ag. Food Chem. (in  
15 press). Thioredoxin *h* was purified from wheat germ (Florencio, F.J., et al. (1988) Arch Biochem. Biophys. 266:496-507) and thioredoxins *f* and *m* from spinach leaves (Florencio, F.J., et al., *supra.*). Human thioredoxin was a kind gift of Dr. Emanuelle Wollman.  
20 NADP-malate dehydrogenase was purified from corn leaves (Jacquot, J.-P., et al. (1981) Plant Physiol. 68:300-304) and glutathione reductase from spinach leaves (Florencio, F.J. et al., *supra.*). *E. coli* glutaredoxin was a kind gift of Professor A. Holmgren.

#### 25 SDS-Polyacrylamide Gel Electrophoresis

- SDS-polyacrylamide gel electrophoresis was performed in 10-20% gradient gels of 1.5 mm thickness that were developed for 3 hr at a constant current of 40 mA. Following electrophoresis, gels were soaked for 2 hr in  
30 12% (w/v) trichloroacetic acid and then transferred to a solution containing 40% methanol and 10% acetic acid for 12 hr to remove excess mBBR. The fluorescence of protein-bound mBBR was determined by placing gels on a light box fitted with an ultraviolet light source (365

nm). Gels were photographed with Polaroid positive/negative Landfilm, type 55, through a yellow Wratten gelatin filter No. 8 (cutoff=460 nm) (exposure time 40 sec. at *f*4.5). Gels were stained for protein for 1 hr  
5 in solution of 0.125% (w/v) Coomassie blue R-250 in 10% acetic acid and 40% methanol. Gels were destained in this same solution from which Coomassie blue was omitted.

Polaroid negatives of fluorescent gels and dry stained  
10 gels were scanned with a laser densitometer (Pharmacia-LKB Ultrosan XL). The bands were quantified by evaluating areas or height of the peaks with Gelscan XL software.

#### EXAMPLE 27

#### 15      Reduction of Toxins and Labeling with mBBR

Reactions were carried out with 10  $\mu$ g of target toxin in a final volume of 100  $\mu$ l in 30 mM Tris-HCl buffer, pH 7.9, with 0.8  $\mu$ g thioredoxin, 0.7  $\mu$ g NTR (both from *E. coli*) and 2.5 mM NADPH. When thioredoxin was reduced  
20 by DTT, NADPH and NTR were omitted and DTT was added to 0.5 mM. Assays with GSH were performed similarly, but at a final concentration of 1 mM. For reduction by glutaredoxin, the thioredoxin and NTR were replaced by 1  $\mu$ g *E. coli* glutaredoxin, 0.38  $\mu$ g glutathione reductase  
25 (partially purified from spinach leaves), 1 mM GSH and 2.5 mM NADPH (the combination of these four components is called NADP/glutaredoxin system). Reduction by the reduced form of lipoic acid, was carried out in a volume of 100  $\mu$ l at two concentrations, 100  $\mu$ M and 200  $\mu$ M, both  
30 alone and with 0.8  $\mu$ g of thioredoxin. The mixture was incubated for 2 hr at 37C in the case of erabutoxin b and  $\alpha$ -bungarotoxin, 1 hr at room temperature for  $\beta$ -bungarotoxin and 20 min at room temperature for phospholipase A<sub>2</sub>. After incubation, mBBR was added to

1.5 mM and the reaction continued for 15 min at room temperature. The reaction was stopped and excess mBBr derivatized by adding 10  $\mu$ l of 100 mM  $\beta$ -mercaptoethanol, 5  $\mu$ l of 20 % SDS and 10  $\mu$ l 50 % glycerol. Samples were  
5 then analyzed by SDS-polyacrylamide gel electrophoresis.

Total toxin reduction was accomplished by boiling samples for 3 min in 2 mM DTT. After cooling, the samples were labeled with mBBr and treated as before, except that all samples were again boiled for 2 min  
10 prior to loading in the gel. The extent of the reduction of erabutoxin b by the different reductants described above is shown in Fig. 49. Dithiothreitol (DTT) and the reduced forms of thioredoxin and lipoic acid are dithiol reductants as opposed to monothiol  
15 reductants like 2-mercaptoethanol and glutathione. DTT is a synthetically prepared chemical agent, whereas thioredoxin and lipoic acid occur within the cell. Evidence presented above demonstrates that lipoic acid is a more specific reductant than dithiothreitol.  
20 Dithiothreitol reduced the toxin partly without thioredoxin (lane 5) whereas reduced lipoic acid did not (lane 8). Fig. 52 shows that the NTS or DTT plus thioredoxin are specific reductants for  $\alpha$ -bungarotoxin and  $\beta$ -bungarotoxin.

25

#### EXAMPLE 28

##### NADP-Malate Dehydrogenase Activation

The ability of snake toxins to activate chloroplast NADP-malate dehydrogenase was carried out by preincubating 5  $\mu$ g toxin with a limiting thioredoxin  
30 concentration (to restrict activation of the enzyme by the thioredoxin): *E. coli* thioredoxin, 0.25  $\mu$ g; human, 0.9  $\mu$ g; wheat, 1.15  $\mu$ g; spinach *f* and *m*, 0.375 and 0.125  $\mu$ g, respectively. Purified corn NADP-malate

dehydrogenase, 1.4  $\mu$ g, was added to a solution containing 100 mM Tris-HCl, pH 7.9, thioredoxin as indicated, and 10 mM DTT (final volume 0.2 ml). After 25 min, 160  $\mu$ l of the preincubation mixture was injected  
5 into a 1 cm cuvette of 1 ml capacity containing (in 0.79 ml) 100 mM Tris HCl, pH 7.9, and 0.25 mM NADPH. The reaction was started by the addition of 50  $\mu$ l of 50 mM oxalacetic acid. NADPH oxidation was followed by monitoring the change in absorbance at 340 nm with a  
10 Beckman spectrophotometer fitted with a four-way channel changer. Fig. 50 which represents the results of this experiment shows that the reduction by different reduced thioredoxins of erabutoxin b significantly alters the toxin's biological ability to activate NADP-malate  
15 dehydrogenase. The results demonstrate that, although there are differences in effectiveness, all thioredoxins tested function to some extent in limiting the effect of the toxin.

#### EXAMPLE 29

#### 20 Proteolysis assay of Erabutoxin b

Erabutoxin b, 10  $\mu$ g was incubated for 2 hr at 37C with 30 mM Tris-HCl buffer pH 7.9 (total volume, 100  $\mu$ l). As indicated, the buffer was supplemented with 0.8  $\mu$ g thioredoxin, 0.7  $\mu$ g NTR and 2.5 mM NADPH. When  
25 thioredoxin was reduced by DTT the NTR and NADPH were omitted and DTT was added to 0.5 mM. Following incubation, samples were digested with 0.4 and 2  $\mu$ g of trypsin for 10 min at 37 C. DTT, 4.8  $\mu$ l of 50 mM solution, 5  $\mu$ l of 20% SDS and 10  $\mu$ l of 50% glycerol were  
30 added, samples were boiled for 3 min, and then subjected to SDS-polyacrylamide gel electrophoresis. Gels were stained with Coomassie blue and the protein bands quantified by densitometric scanning as described above. The results of the assay are shown in Table XVI below.

These results show that reduction of a snake neurotoxin (erabutoxin b) renders the toxin more susceptible to proteolysis. An extension of this conclusion would indicate that administration of reduced thioredoxin as a toxin antidote should help to destroy the toxin owing to the increase in proteolytic inactivation by proteases of the venom.

TABLE XVI

Susceptibility of the Oxidized and Reduced Forms of Erabutoxin b to Trypsin

| Treatment          | % Erabutoxin b digested              |                                    |
|--------------------|--------------------------------------|------------------------------------|
|                    | <u>0.4 <math>\mu</math>g trypsin</u> | <u>2 <math>\mu</math>g trypsin</u> |
| Control            | 0.0                                  | 34.1                               |
| Reduced, NTS       | 21.1                                 | 57.8                               |
| 15 Reduced, DTT    | 3.1                                  | 40.6                               |
| Reduced, DTT + Trx | 28.0                                 | 71.8                               |

Erabutoxin b, 10  $\mu$ g was preincubated for 2 hours at 37C in 30 mM Tris-HCl buffer, pH 7.9, as follows: control, no addition; reduced by *E. coli* NADP/thioredoxin system (NTS), thioredoxin, NTR and NADPH; reduced by DTT, DTT; and reduced by DTT plus thioredoxin, DTT supplemented with *E. coli* thioredoxin. After preincubation 0.4  $\mu$ g and 2  $\mu$ g of trypsin were added to the indicated which then were analyzed by SDS-polyacrylamide gel electrophoresis.

## EXAMPLE 30

Phospholipase A<sub>2</sub> Assay

Activity of the oxidized and reduced forms of the phospholipase A<sub>2</sub> component of  $\beta$ -bungarotoxin was determined spectrophotometrically following change in acidity as described by Lobo de Araujo, et al. (1987) Toxicon 25:1181-1188. For reduction experiments, 10  $\mu$ g

toxin was incubated in 30 mM Tris-HCl buffer, pH 7.9, containing 0.8  $\mu$ g thioredoxin, 0.7  $\mu$ g NTR and 7 mM NADPH (final volume, 35  $\mu$ l). After 1 hr incubation at room temperature, 20  $\mu$ l of the reaction mixture was added to  
5 a 1 cm cuvette containing 1 ml of assay solution (adjusted to pH 7.6) that contained 10 mM  $\text{CaCl}_2$ , 100 mM NaCl, 4 mM sodium cholate, 175  $\mu$ M soybean phosphatidylcholine and 55  $\mu$ M phenol red. The reaction was followed by measuring the change in the absorbance  
10 at 558 nm in a Beckman Du model 2400 spectrophotometer. The results of this experiment which are shown in Figure 51, demonstrate that  $\beta$ -bungarotoxin loses most of its phospholipase activity when reduced by thioredoxin. The results are consistent with the conclusion that the  
15 administration of reduced thioredoxin following a snake bite would help detoxify the toxin by eliminating phospholipase  $A_2$  activity.

#### EXAMPLE 31

##### $\alpha$ -Bungarotoxin binding to Acetylcholine Receptor

20  $\alpha$ -Bungarotoxin binding was assayed with cultured mouse cells by using radiolabeled toxin (Gu, Y., et al. (1985) J. Neurosci. 5:1909-1916). Mouse cells, line  $C_2$ , were grown as described by Gu et al (Gu, Y. et al., supra.) and plated in 24-well plastic tissue culture plates  
25 (Falcon) at a density of about 3000 cells per well. Growth medium was replaced by fusion medium after 48 hr and again after 96 hr. Cultures were used for assay after an additional 2 days growth.

$\alpha$ -Bungarotoxin binding was determined with cells  
30 subjected to three different treatments: [A] 10 nM  $\alpha$ -bungarotoxin $^{125}\text{I}$  (262 Ci/mmol) was preincubated 2 hr at 37°C in 200  $\mu$ l of phosphate-saline buffer (0.15M NaCl in 10 mM  $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$  pH 7.2) with 4  $\mu$ g thioredoxin,

3.5  $\mu$ g NTR (both from *E. coli*) and 6.25 mM NADPH. In certain cases, the NTR and NADPH were replaced by 1.25 mM DTT. After 2 hr incubation, the mixture was transferred to a well containing mouse cells, washed two times with phosphate-saline, and incubated for 2 hours at 37°C. [B] After washing the cells two times with phosphate-saline buffer, 10 nM  $\alpha$ -bungarotoxin<sup>125</sup>I (in 200  $\mu$ l of phosphate-saline) was added per well. Following a 2 hr incubation at 37°C, cells were washed again with phosphate-saline buffer to remove unbound toxin. As indicated, 200  $\mu$ l saline, supplemented with 0.68 mM CaCl<sub>2</sub>, 0.49 mM MgCl<sub>2</sub>, 4  $\mu$ g thioredoxin, 3.5  $\mu$ g NTR and 6.25 mM NADPH were added to the well. The plate was incubated 2 hr at 37°C. NTR and NADPH were omitted from treatment with DTT which was added at 1.25 mM. [C] After washing cells twice with phosphate-saline buffer, 200  $\mu$ l of a solution containing 4  $\mu$ g thioredoxin, 3.5  $\mu$ g NTR and 6.25 mM NADPH, were added to each well. In some cases, NTR and NADPH were replaced with 1.25 mM DTT. The plate was incubated for 2 hr at 37°C. Cells were then washed twice with phosphate-saline buffer to remove the added reductant. Phosphate-saline buffer, 200  $\mu$ l, containing 0.68 mM CaCl<sub>2</sub> and 0.49 mM MgCl<sub>2</sub> and 10 nM  $\alpha$ -bungarotoxin<sup>125</sup>I was added to each well. Incubation was continued for 2 hr at 37°C. The results of this assay are shown in Table XVII. This experiment shows that when reduced by thioredoxin,  $\beta$ -bungarotoxin can no longer bind to the acetylcholine receptor. When extended to the whole animal, the thioredoxin-linked reduction mechanism would result in detoxification by eliminating binding of the toxin to its target receptor.

Each  $\alpha$ -bungarotoxin binding assay was done in triplicate. Nonspecific binding was measured by adding 100-fold excess unlabeled  $\alpha$ -bungarotoxin to the incubation mixture. After the incubation period, the

cells in all cases were washed with phosphate-saline to remove unbound toxin. The amount of toxin bound was determined by solubilizing the cells in 0.1 M NaOH and measuring radioactivity in a gamma counter.

5

TABLE XVII

Binding of  $\alpha$ -Bungarotoxin to the  
Acetylcholine Receptor of Mouse Cells

|                    |  | <u>%Binding</u> |  |
|--------------------|--|-----------------|--|
| <u>Treatment A</u> |  |                 |  |
| 10                 | Toxin + Reductant $\xrightarrow{2 \text{ hr}, 37^\circ\text{C}}$ cells $\xrightarrow{2 \text{ hr}, 37^\circ\text{C}}$ STOP                       |                 |  |
|                    | No reductant   | 100.0           |  |
|                    | NTS  | 0.0             |  |
|                    | DTT plus Thioredoxin   | 0.0             |  |
| 15                 | NTS minus NTR  | 63.0            |  |
|                    | NTS minus Thioredoxin  | 78.0            |  |
|                    | NTS minus NADPH  | 101.0           |  |
| <u>Treatment B</u> |  |                 |  |
| 20                 | Toxin + Cells $\xrightarrow{2 \text{ hr}, 37^\circ\text{C}}$ wash cells $\xrightarrow[+ \text{ reductant}]{2 \text{ hr}, 37^\circ\text{C}}$ STOP |                 |  |
|                    | No reductant   | 100.0           |  |
|                    | NTS  | 78.0            |  |
|                    | DTT plus Thioredoxin   | 76.0            |  |
| 25                 | <u>Treatment C</u>   |                 |  |
|                    | Cells + Reductant $\xrightarrow{2 \text{ hr}, 37^\circ\text{C}}$ wash cells $\xrightarrow[+ \text{ toxin}]{2 \text{ hr}, 37^\circ\text{C}}$ STOP |                 |  |
|                    | No reductant   | 100.0           |  |
| 30                 | NTS  | 68.7            |  |
|                    | DTT  | 85.0            |  |
|                    | DTT plus Thioredoxin   | 68.8            |  |

*E. coli* NTS:thioredoxin, NTR and NADPH

EXAMPLE 32Example for Detoxification in an Animal

Detoxification of snake neurotoxins is determined by subcutaneous injection into mice. Assays are done in triplicate. Prior to injection, the toxin is diluted in phosphate-saline buffer (0.15M NaCl in 10 mM  $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$  pH 7.2) at concentrations ranging up to twice the  $\text{LD}_{50}$  dose. ( $\text{LD}_{50}$  is defined as that dose of toxin that kills 50% of a given group of animals.) For toxicity tests, the following neurotoxin concentrations correspond to the  $\text{LD}_{50}$  (per g mouse): erabutoxin b, 0.05  $\mu\text{g}$  - 0.15  $\mu\text{g}$ ;  $\alpha$ -bungarotoxin, 0.3  $\mu\text{g}$ ; and  $\beta$ -bungarotoxin, 0.089  $\mu\text{g}$ . At 5, 10, 30, 60 minutes and 4, 12 and 24 hr after injection, separate groups of the challenged mice are injected (1) intravenously, and (2) subcutaneously (multiple local injections around the initial injection site). The thioredoxin is reduced with: (1) the *E. coli* NADP-thioredoxin system, using 0.08  $\mu\text{g}$  thioredoxin, 0.07  $\mu\text{g}$  NTR and 25 nanomoles NADPH; (2) Thioredoxin plus 1-2 nanomoles of reduced lipoic acid, using 0.08  $\mu\text{g}$  *E. coli* or 0.20  $\mu\text{g}$  human thioredoxin, and (3) using 0.08  $\mu\text{g}$  *E. coli* or 0.20  $\mu\text{g}$  human thioredoxin with 5 nanomoles dithiothreitol (concentrations are per  $\mu\text{g}$  toxin injected into the animal; all solutions are prepared in phosphate-saline buffer).

The effect of thioredoxin on detoxification is determined by (1) comparing the  $\text{LD}_{50}$  with the control group without thioredoxin; (2) following the extent of the local reaction, as evidenced by necrosis, swelling and general discomfort to the animal; (3) following the serum levels of creatin kinase, an indicator of tissue damage. Creatin kinase, which is released into the blood as a result of breakage of muscle cells, is

monitored using the standard assay kit obtained from Sigma Chemical Co. (St. Louis, MO).

The symptoms of snake bite are multiple and depend on a variety of factors. As a consequence, they vary from patient to patient. There are, nonetheless, common symptoms that thioredoxin treatment should alleviate in humans. Specifically, the thioredoxin treatment should alleviate symptoms associated with neurotoxic and related effects resulting from snake bite. Included are a decrease in swelling and edema, pain and blistering surrounding the bite; restoration of normal pulse rate; restriction of necrosis in the bite area; minimization of the affected part. A minimization of these symptoms should in turn result in improvement in the general health and state of the patient.

#### CONCLUDING REMARKS

It can be seen from the foregoing general description of the invention and from the specific examples illustrating applications thereof, that the invention has manifold and far reaching consequences. The invention basically provides novel dough and dough mixtures and novel methods for creating new doughs and for improving the quality of dough and baked goods as well as novel methods for inactivating enzyme inhibitors in cereal products. The invention also provides a novel method for altering the biological activity and inactivity of animal toxins, namely bee, scorpion and snake toxins. The invention further provides a novel protein that is a pullulanase inhibitor and a method for its inactivation.

While the invention has described in connection with certain specific embodiments thereof, it should be realized that various modifications as may be apparent to those of skill in the art to which the invention  
5 pertains also fall within the scope of the invention as defined by the appended claims.

WHAT IS CLAIMED IS:

1. A method of reducing a cystine containing, non-thionin protein comprising
  - (a) adding a thiol redox protein to a liquid or  
5 substance containing said cystine containing protein;
  - (b) reducing said thiol redox protein, and
  - (c) reducing said cystine containing protein by  
said reduced thiol redox protein.
2. The method of Claim 1 wherein the thiol redox  
10 protein is thioredoxin.
3. The method of Claim 2 wherein the thiol redox protein is reduced by NADPH and NADP-thioredoxin reductase.
4. The method of Claim 1 wherein the thiol redox  
15 protein is glutaredoxin.
5. A composition comprising a cystine containing, non-thionin protein, thioredoxin, NADP-thioredoxin reductase and NADPH or an NADPH generator composition.
6. A method of reducing an amylase inhibitor protein  
20 having disulfide bonds comprising
  - (a) adding a thiol redox protein to a liquid or substance containing said amylase inhibitor,
  - (b) reducing said thiol redox protein; and
  - (c) reducing said inhibitor protein by said  
25 reduced thiol redox protein.
7. The method of Claim 6 wherein the thiol redox protein is thioredoxin.

8. The method of Claim 7 wherein the thiol redox protein is reduced by NADP-thioredoxin reductase in combination with NADPH.
9. The method of Claim 6 wherein the amylase inhibitor protein is a CM or DSG protein.
10. The method of Claim 6 wherein the thiol redox protein is glutaredoxin.
11. The method of Claim 10 wherein glutaredoxin is reduced by reduced glutathione.
- 10 12. The method of Claim 10 wherein the amylase inhibitor protein is the  $\alpha$ -amylase inhibitor CM-1, DSG-1 or DSG-2.
- 15 13. A composition comprising an amylase inhibitor protein having disulfide bonds, a thioredoxin, NADP-thioredoxin reductase and NADPH or an NADPH generator composition.
14. A method of reducing a protease inhibitor protein having disulfide bonds comprising
  - (a) adding a thiol redox protein to a liquid or substance containing said protease inhibitor;
  - 20 (b) reducing said thiol redox protein by a reductant or reduction system, and
  - (c) reducing said protease inhibitor by said reduced thiol redox protein.
- 25 15. The method of Claim 14 wherein the thiol redox protein is thioredoxin.
16. The method of Claim 15 wherein the reductant is lipoic acid.

17. The method of Claim 15 wherein the reduction system is NADP-thioredoxin reductase and NADPH.
18. The method of Claim 15 wherein the protease inhibitor protein is a trypsin inhibitor.
- 5 19. The method of Claim 18 wherein the trypsin inhibitor is selected from the group consisting of corn kernel, ovoinhibitor, ovomucoid, aprotonin, Bowman-Birk and Kunitz trypsin inhibitor.
- 10 20. The method of Claim 14 wherein the thiol redox protein is glutaredoxin.
21. The method of Claim 20 wherein the reductant for reducing glutaredoxin is reduced glutathione.
- 15 22. The method of Claim 20 wherein the protease inhibitor protein is a trypsin inhibitor protein selected from the group consisting of corn kernel and ovoinhibitor trypsin inhibitor.
23. The method of Claim 14 wherein the protease inhibitor protein is a subtilisin inhibitor.
- 20 24. A method of reducing a gliadin containing a cystine group comprising
- (a) adding a thiol redox protein to a liquid or substance containing said gliadin;
  - (b) reducing said thiol redox protein by a reductant or reduction system, and
  - 25 (c) reducing said gliadin by said reduced thiol redox protein.
25. The method of Claim 24 wherein the thiol redox protein is thioredoxin.

26. The method of Claim 24 wherein the reduction system is NADP-thioredoxin reductase and NADPH.
27. The method of Claim 24 wherein the gliadin protein is selected from the group consisting of the  $\alpha$ ,  $\beta$  and  $\gamma$  protein type.
28. The method of Claim 24 wherein the thiol redox protein is glutaredoxin.
29. The method of Claim 28 wherein the reductant for reducing glutaredoxin is reduced glutathione.
30. The method of Claim 28 wherein the gliadin is an  $\alpha$ ,  $\beta$  or  $\gamma$  gliadin.
31. A method of reducing a glutenin comprising
- (a) adding a thiol redox protein to a liquid or substance containing said glutenin;
  - (b) reducing said thiol redox protein by a reductant or a reduction system, and
  - (c) reducing said glutenin by said reduced thiol redox protein.
32. The method of Claim 31 wherein the thiol redox protein is thioredoxin.
33. The method of Claim 31 wherein the reduction system is NADPH and NADP-thioredoxin reductase.
34. The method of Claim 31 wherein the glutenin has a molecular mass of from about 30 to about 130 kDa.
35. The method of Claim 31 wherein the thiol redox protein is glutaredoxin.

36. The method of Claim 35 wherein the reductant for reducing glutaredoxin is reduced glutathione.
37. The method of Claim 35 wherein the glutenin has a molecular mass of from about 30 to about 130 kDa.
- 5 38. A method for inactivating an enzyme inhibitor protein in a food product, said inhibitor containing cystine groups, comprising
- (a) mixing said cereal grain product with a thiol redox protein, said thiol redox protein being alone or
  - 10 in combination with a reductant or a reduction system,
  - (b) reducing said thiol redox protein, and
  - (c) reducing said enzyme inhibitor by said reduced thiol redox protein, said reduction of said inhibitor causing said inhibitor to be inactivated.
- 15 39. The method of Claim 38 wherein the food product is soybean and the inhibitor is a trypsin inhibitor.
40. The method of Claim 38 wherein the thiol redox protein is thioredoxin.
41. The method of Claim 38 wherein said grain is
- 20 barley and said inhibitor is a barley amylase/subtilisin (asi) inhibitor, a CM protein or a DSG protein.
42. A composition comprising thioredoxin, NADP-thioredoxin reductase, NADPH or an NADPH generator
- 25 composition.
43. A method of improving the characteristics of a dough or a baked good comprising the steps of:
- (a) mixing a thiol redox protein with dough ingredients containing glutenins or gliadins to form a

dough, said thiol redox protein being alone or in combination with a reductant or a reduction system; and

(b) baking the dough to form a baked good.

44. The method of Claim 43 wherein the thiol redox  
5 protein is thioredoxin.

45. The method of Claim 43 wherein thioredoxin comprises from about 0.01 to about 0.3 ppm of said dough.

46. A method of improving the characteristics of a  
10 semolina dough or cooked pasta comprising the steps of:

(a) mixing a thiol redox protein with semolina dough ingredients containing glutenins or gliadins to form a dough, said thiol redox protein being alone or in combination with a reductant or a reduction system;

15 (b) shaping the dough mixture from step (a), and

(c) cooking the shaped dough mixture from step (b) to form a cooked pasta.

47. The method of Claim 46 wherein the thiol redox protein is thioredoxin.

20 48. A method of producing a dough from rice, corn, soybean, barley, oat, sorghum, cassava or millet flour, comprising

(a) mixing a thiol redox protein with said flour to form a mixture, said flour containing storage  
25 proteins;

(b) reducing said thiol redox protein in said mixture;

(c) reducing said storage proteins by said reduced thiol redox protein, and

30 (d) oxidizing said reduced storage proteins, said oxidized storage proteins creating a protein network complex in the form of a pliable dough.

49. A method of producing a dough from rice, corn, soybean, barley, oat, sorghum or millet flour, comprising

5 (a) mixing a reduced thiol redox protein with said flour and a liquid to form a mixture, said flour containing water insoluble storage proteins;

(b) reducing said storage proteins by said reduced thiol redox protein, and

10 (c) oxidizing said reduced storage proteins, said oxidized storage proteins creating a protein network complex in the form of a pliable dough.

50. A method for producing an improved gluten comprising

15 (a) mixing a wheat or rye flour with a liquid to form a mixture, said flour containing glutenins, gliadins and cystine containing soluble proteins;

(b) adding a thiol redox protein;

(c) reducing said thiol redox protein by a reductant or reduction system;

20 (d) reducing said gliadins, glutenins and soluble proteins by said reduced thiol redox protein, said reduced glutenins, gliadins and soluble proteins forming gluten, and

(e) separating said gluten from said mixture.

25 51. A method for producing an improved gluten comprising

(a) mixing a wheat or rye flour with a reduced thiol redox protein and a liquid, said flour containing glutenins or gliadins;

30 (b) reducing said gliadins and glutenins by said reduced thiol redox protein, said reduced glutenins and gliadins forming gluten, and

(c) separating said gluten from said mixture.

52. A method for producing a gluten like product comprising

(a) mixing a barley, corn, sorghum, rice or millet flour with a liquid to form a mixture, said  
5 flour containing water insoluble storage proteins and cystine containing soluble proteins;

(b) adding a thiol redox protein to said mixture;

(c) reducing said thiol redox protein by a reductant or reduction system;

10 (d) reducing said water insoluble storage proteins and soluble proteins by said reduced thiol redox protein, said reduced proteins forming a sticky, elastic gluten like product, and

(e) separating said gluten like product from said  
15 mixture.

53. A dough mixture comprising a thiol redox protein, said thiol redox protein being from about 0.1 ppm to about 1.0 ppm of said mixture.

54. A yeast cell transformed with a vector containing  
20 a recombinant thioredoxin DNA.

55. The cell of Claim 54 wherein said thioredoxin DNA is expressed to produce thioredoxin.

56. The cell of Claim 55 wherein said thioredoxin is secreted.

25 57. A yeast cell transformed with a vector containing a recombinant NADP-thioredoxin reductase DNA.

58. The cell of Claim 57 wherein said reductase DNA is expressed to produce said reductase.

59. The cell of Claim 58 wherein said reductase is secreted.
60. A lysed and freeze-dried cell of Claim 55.
61. A lysed and freeze-dried cell of Claim 58.
- 5 62. A method for improving dough quality comprising  
(a) mixing lysed yeast cells which express  
thioredoxin and lysed yeast cells which express NADP-  
thioredoxin reductase with NADPH or an NADPH generator  
and water or a liquid buffer to form a mixture, and  
10 (b) adding said mixture to flour to form a dough.
63. A method for improving dough quality comprising  
(a) mixing lysed yeast cells which express  
thioredoxin and lysed yeast cells which express NADP-  
thioredoxin reductase with NADPH or an NADPH generator  
15 to form a mixture, and  
(b) adding said mixture to dough ingredients to  
form a dough.
64. A method for improving the quality of a baked good  
comprising  
20 (a) mixing lysed yeast cells which express  
thioredoxin and lysed yeast cells which express NADP-  
thioredoxin reductase with NADPH or an NADPH generator  
and water or a liquid buffer to form a mixture;  
(b) adding said mixture to flour to form a dough,  
25 and  
(c) baking said dough to produce a baked good.
65. A method for improving the quality of a baked good  
comprising  
(a) mixing lysed yeast cells which express  
30 thioredoxin and lysed yeast cells which express NADP-

thioredoxin reductase with NADPH or an NADPH generator to form a mixture, and

(b) adding said mixture to dough ingredients to form a dough, and

5 (c) baking said dough to produce a baked good.

66. A method of reducing the intramolecular disulfide bonds of a non-thionin, non-chloroplast protein containing more than one intramolecular cystine comprising:

10 (a) adding a thiol redox protein to a liquid or substance containing said cystine containing protein;

(b) reducing said thiol redox protein, and

(c) reducing said cystine containing protein by said reduced thiol redox protein.

15 67. The method of Claim 66 wherein the thiol redox protein is thioredoxin.

68. The method of Claim 66 wherein the thiol redox protein is reduced by NADPH and NADP-thioredoxin reductase.

20 69. The method of Claim 66 wherein the thiol redox protein is reduced by an NADPH generator composition.

70. A composition comprising an intramolecular cystines containing non-thionin, non-chloroplast, plant protein, thioredoxin, NADP-thioredoxin reductase and  
25 NADPH or an NADPH generator composition.

71. A method of decreasing the heat or protease stability of a protein having intramolecular disulfide bonds comprising:

(a) adding a thiol redox protein to a liquid or  
30 substance containing said protein having said intramolecular disulfide bonds;

- (b) reducing said thiol redox protein; and
- (c) reducing said intramolecular disulfide bonds by said reduced thiol redox protein.

72. The method of Claim 71 wherein the thiol redox  
5 protein is thioredoxin.

73. The method of Claim 71 wherein the thiol redox protein is reduced by NADPH and NADP-thioredoxin reductase or an NADPH generator composition.

74. A method of selectively substantially reducing  
10 only the intramolecular disulfide bonds of a particular protein having intramolecular and intermolecular disulfide bonds comprising:

- (a) adding a thiol redox protein to a liquid or substance containing said particular protein;
- 15 (b) reducing said thiol redox protein by a reductant or reduction system thereby substantially reducing only said intramolecular disulfide bonds of said particular protein by said reduced thiol redox protein.

20 75. The method of Claim 74 wherein the thiol redox protein is thioredoxin.

76. The method of Claim 74 wherein the protein having the intramolecular disulfide bonds is a 2S albumin protein.

25 77. The method of Claim 75 wherein the reduced thioredoxin was reduced with NADPH and NTR or with DTT.

78. An isolated pullulanase inhibitor protein having disulfide bonds and a molecular weight of between 8 to 15 kDa.

79. A method of inactivating the pullulanase inhibitor activity of the inhibitor protein of Claim 78 comprising:

- 5 (a) adding thioredoxin to a liquid or substance containing said protein;
- (b) reducing said thioredoxin; and
- (c) reducing said inhibitor protein by said reduced thioredoxin.

80. A method of increasing the activity of pullulanase derived from barley or wheat endosperm comprising:

- 10 (a) adding thioredoxin to a liquid or substance containing said pullulanase; and
- (b) reducing said thioredoxin thereby increasing said pullulanase activity.

15 81. A method of improving the characteristics of a cooked pasta comprising the steps of:

- (a) mixing a thiol redox protein with pasta dough ingredients to form a dough, said thiol redox protein being alone or in combination with a reductant or a  
20 reduction system;
- (b) shaping the dough mixture from step (a), and
- (c) cooking the shaped dough mixture from step (b) to form a cooked pasta.

25 82. A method of improving the characteristics of a dough or a baked good comprising the steps of:

- (a) mixing NADPH or an NADPH generator composition with dough ingredients containing glutenins or gliadins to form a dough, and
- (b) baking the dough to form a baked good.

30 83. A method of improving the characteristics of a baked good comprising the steps of:

- (a) mixing a thiol redox protein with the dough ingredients to form a dough, said thiol redox protein

being alone or in combination with a reductant or a reduction system;

(b) shaping the dough mixture from step (a), and

(c) baking the shaped dough mixture from step (b)

5 to form a baked good.

84. A method of improving the characteristics of a triticale baked good comprising the steps of:

(a) mixing a liquid and thioredoxin with triticale flour to form a dough mixture, said thio  
10 redoxin being in combination with NTR and an NADPH generating system; and

(b) baking the dough to form a baked good.

85. A method of reducing a snake neurotoxin protein having one or more intramolecular cystines comprising:

15 (a) contacting said cystine containing protein with an amount of a thiol redox (SH) agent effective for reducing said protein, and

(b) maintaining said contact for a time sufficient to reduce one or more disulfide bridges of  
20 said one or more intramolecular cystines thereby reducing said neurotoxin protein.

86. The method of Claim 85 wherein the thiol redox (SH) agent is selected from the group consisting of a reduced thioredoxin, reduced lipoic acid in the  
25 presence of a thioredoxin, DTT and DTT in the presence of a thioredoxin.

87. The method of Claim 85 wherein the snake neurotoxin protein is a presynaptic neurotoxin.

88. The method of Claim 87 wherein the presynaptic  
30 neurotoxin protein is a  $\beta$ -neurotoxin.

89. The method of Claim 88 wherein the  $\beta$ -neurotoxin is  $\beta$ -bungarotoxin.
90. The method of Claim 89 wherein the presynaptic neurotoxin is a facilitatory neurotoxin.
- 5 91. The method of Claim 85 wherein the snake neurotoxin is a postsynaptic neurotoxin.
92. The method of Claim 91 wherein the postsynaptic neurotoxin is a short neurotoxin or a long neurotoxin.
93. The method of Claim 92 wherein the postsynaptic  
10 neurotoxin is the short neurotoxin, erabutoxin a or erabutoxin b.
94. The method of Claim 92 wherein the postsynaptic neurotoxin is the long neurotoxin,  $\alpha$ -bungarotoxin or  $\alpha$ -cobratoxin.
- 15 95. A reduced snake neurotoxin protein prepared according to the method of Claim 85.
96. A composition comprising a snake neurotoxin protein and a thiol redox (SH) agent.
97. A method of reducing a snake neurotoxin protein  
20 having one or more intramolecular cystines comprising:  
    (a) contacting said protein with amounts of NADP-thioredoxin reductase, NADPH or an NADPH generator system and a thioredoxin effective for reducing said toxin, and  
25      (b) maintaining said contact for a time sufficient to reduce one or more disulfide bridges of said one or more intramolecular cystines thereby reducing said protein.

98. A reduced snake neurotoxin prepared according to the method of Claim 97.

99. A method of altering the biological activity of a snake neurotoxin having one or more intramolecular  
5 cystines comprising

(a) contacting a medium containing said neurotoxin with an amount of a thiol redox (SH) agent sufficient for reducing said toxin, and

(b) maintaining said contact for a time  
10 sufficient to reduce one or more disulfide bridges of said one or more intramolecular cystines thereby altering said biological activity of said toxin.

100. The method of Claim 99 wherein the thiol redox (SH) agent is selected from the group consisting of a  
15 reduced thioredoxin, reduced lipoic acid in the presence of a thioredoxin, DTT and DTT in the presence of a thioredoxin.

101. The method of Claim 99 wherein the snake neurotoxin is a presynaptic neurotoxin.

20 102. The method of Claim 101 wherein the presynaptic neurotoxin is the  $\beta$ -neurotoxin,  $\beta$ -bungarotoxin.

103. The method of Claim 101 wherein the presynaptic neurotoxin is a facilitatory neurotoxin.

104. The method of Claim 99 wherein the snake  
25 neurotoxin is a postsynaptic neurotoxin.

105. The method of Claim 104 wherein the postsynaptic neurotoxin is a short neurotoxin or a long neurotoxin.

106. The method of Claim 105 wherein the postsynaptic neurotoxin is the short neurotoxin, erabutoxin b or erabutoxin a.

107. The method of Claim 105 wherein the postsynaptic neurotoxin is the long neurotoxin,  $\alpha$ -bungarotoxin or  $\alpha$ -cobratoxin.

108. A method of inactivating, *in vitro*, a snake neurotoxin having one or more intramolecular cystines comprising adding a thiol redox (SH) agent to a liquid containing said toxin wherein said amount of said agent is effective for reducing said toxin.

109. The method of Claim 108 wherein the thiol redox (SH) agent is selected from the group consisting of a reduced thioredoxin, reduced lipoic acid in the presence of a thioredoxin, DTT and DTT in the presence of a thioredoxin.

110. An inactivated snake neurotoxin prepared according to the method of Claim 108.

111. A composition comprising a snake neurotoxin protein and a thiol redox (SH) agent in a liquid.

112. A method of inactivating, *in vitro*, a snake neurotoxin having one or more intramolecular cystines comprising adding to a liquid containing said toxin amounts of NADP-thioredoxin reductase, NADPH or an NADPH generator system and a thioredoxin effective for reducing said toxin thereby inactivating said toxin.

113. An inactivated snake neurotoxin prepared according to the method of Claim 112.

114. A composition comprising a liquid having an inactivated snake neurotoxin, NADP-thioredoxin reductase, NADPH or an NADPH generator system and a thioredoxin.
- 5 115. A method of treating snake venom neurotoxicity in an individual comprising administering, to an individual suffering from snake venom neurotoxicity amounts of a thiol redox (SH) agent effective for reducing or alleviating said snake venom neurotoxicity.
- 10 116. The method of Claim 115 wherein the thiol redox (SH) agent is selected from the group consisting of a reduced thioredoxin, reduced lipoic acid in the presence of a thioredoxin, DTT and DTT in the presence of a thioredoxin.
- 15 117. The method of Claim 115 wherein the snake venom neurotoxicity is caused by  $\alpha$ -bungarotoxin, erabutoxin b or  $\beta$ -bungarotoxin toxin.
118. A method of treating snake venom neurotoxicity in an individual comprising administering, to an individual suffering from snake venom neurotoxicity, amounts of NADP-thioredoxin reductase, NADPH or an NADPH generator system and a thioredoxin effective for reducing or alleviating said snake venom neurotoxicity.
- 20 119. The method of Claim 118 wherein the snake venom neurotoxicity is caused by  $\alpha$ -bungarotoxin, erabutoxin b or  $\beta$ -bungarotoxin toxin.
120. A method of reducing a bee venom toxic protein having one or more intramolecular cystines comprising:
- 30 (a) contacting said cystine containing toxic protein with an amount of a thiol redox (SH) agent effective for reducing said protein, and

(b) maintaining said contact for a time sufficient to reduce one or more disulfide bridges of said one or more intramolecular cystines thereby reducing said venom protein.

- 5 121. The method of Claim 120 wherein the thiol redox (SH) agent is selected from the group consisting of a reduced thioredoxin, reduced lipoic acid in the presence of a thioredoxin, DTT and DTT in the presence of a thioredoxin.
- 10 122. The method of Claim 120 wherein the bee venom protein is phospholipase A<sub>2</sub>.
123. The method of Claim 120 wherein the bee venom is from *Apis mellifera*.
- 15 124. A method of inactivating, *in vitro*, a bee venom having one or more intramolecular cystines comprising adding a thiol redox (SH) agent to a liquid containing said venom wherein said amount of said agent is effective for reducing said venom.
- 20 125. The method of Claim 124 wherein the thiol redox (SH) agent is selected from the group consisting of a reduced thioredoxin, reduced lipoic acid in the presence of a thioredoxin, DTT and DTT in the presence of a thioredoxin.
- 25 126. A method of treating bee venom toxicity in an individual comprising administering, to an individual suffering from bee venom toxicity amounts of a thiol redox (SH) agent effective for reducing or alleviating said bee venom toxicity.
- 30 127. The method of Claim 126 wherein the thiol redox (SH) agent is selected from the group consisting of a

reduced thioredoxin, reduced lipoic acid in the presence of a thioredoxin, DTT and DTT in the presence of a thioredoxin.

128. A method of reducing a scorpion venom toxic protein having one or more intramolecular cystines comprising:

(a) contacting said cystine containing protein with an amount of a thiol redox (SH) agent effective for reducing said protein, and

10 (b) maintaining said contact for a time sufficient to reduce one or more disulfide bridges of said one or more intramolecular cystines thereby reducing said neurotoxin protein.

129. The method of Claim 128 wherein the thiol redox (SH) agent is selected from the group consisting of a reduced thioredoxin, reduced lipoic acid in the presence of a thioredoxin, DTT and DTT in the presence of a thioredoxin.

130. The method of Claim 128 wherein the scorpion venom protein is neurotoxin.

131. The method of Claim 128 wherein the scorpion venom is from *Androctonus australis*.

132. A method of inactivating, *in vitro*, a scorpion venom toxin having one or more intramolecular cystines comprising adding a thiol redox (SH) agent to a liquid containing said venom wherein said amount of said agent is effective for reducing said toxin.

133. The method of Claim 132 wherein the thiol redox (SH) agent is selected from the group consisting of a reduced thioredoxin, reduced lipoic acid in the

presence of a thioredoxin, DTT and DTT in the presence of a thioredoxin.

134. A method of treating scorpion venom toxicity in an individual comprising administering, to an individual  
5 suffering from scorpion venom toxicity amounts of a thiol redox (SH) agent effective for reducing or alleviating said scorpion venom toxicity.

135. The method of Claim 134 wherein the thiol redox (SH) agent is selected from the group consisting of a  
10 reduced thioredoxin, reduced lipoic acid in the presence of a thioredoxin, DTT and DTT in the presence of a thioredoxin.

136. A method of treating bee venom toxicity in an individual comprising administering, to an individual  
15 suffering from bee venom toxicity, amounts of NADP-thioredoxin reductase, NADPH or an NADPH generator system and a thioredoxin effective for reducing or alleviating said bee venom toxicity.

137. A method of treating scorpion venom toxicity in an  
20 individual comprising administering, to an individual suffering from scorpion venom toxicity, amounts of NADP-thioredoxin reductase, NADPH or an NADPH generator system and a thioredoxin effective for reducing or alleviating said scorpion venom toxicity.

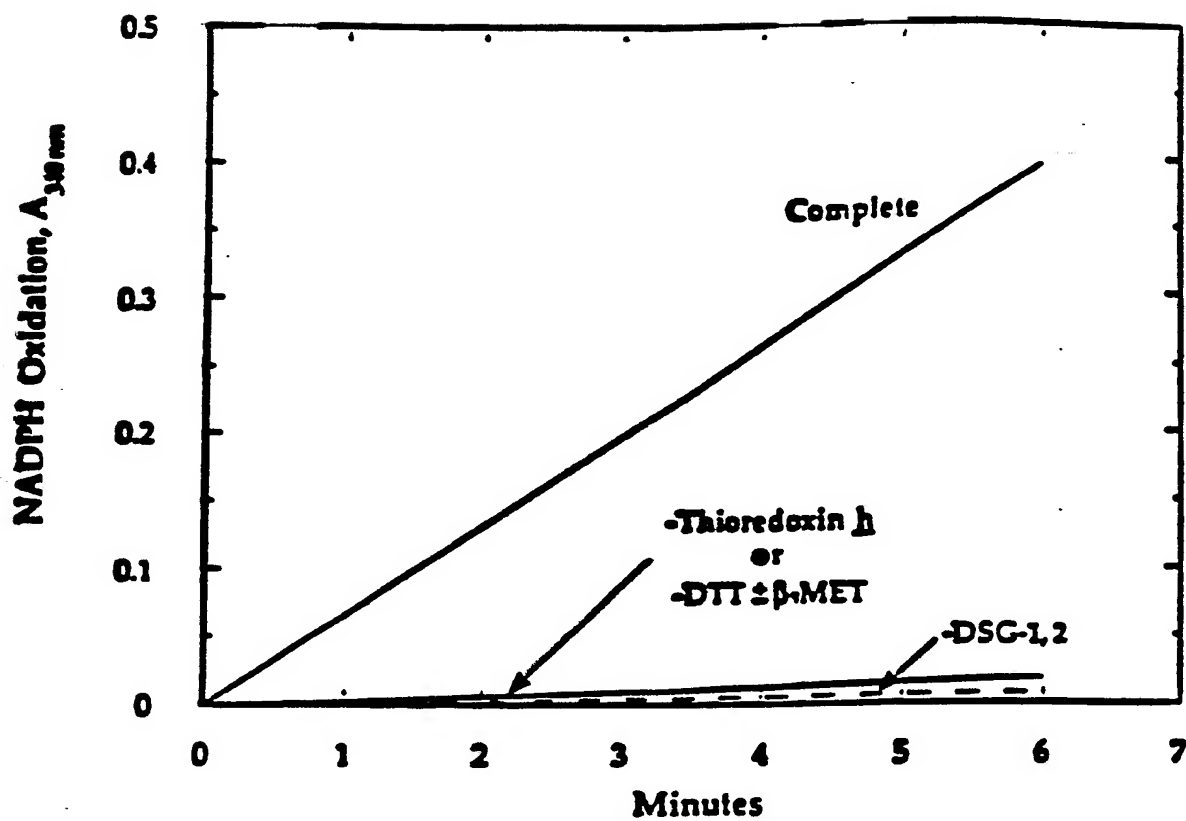
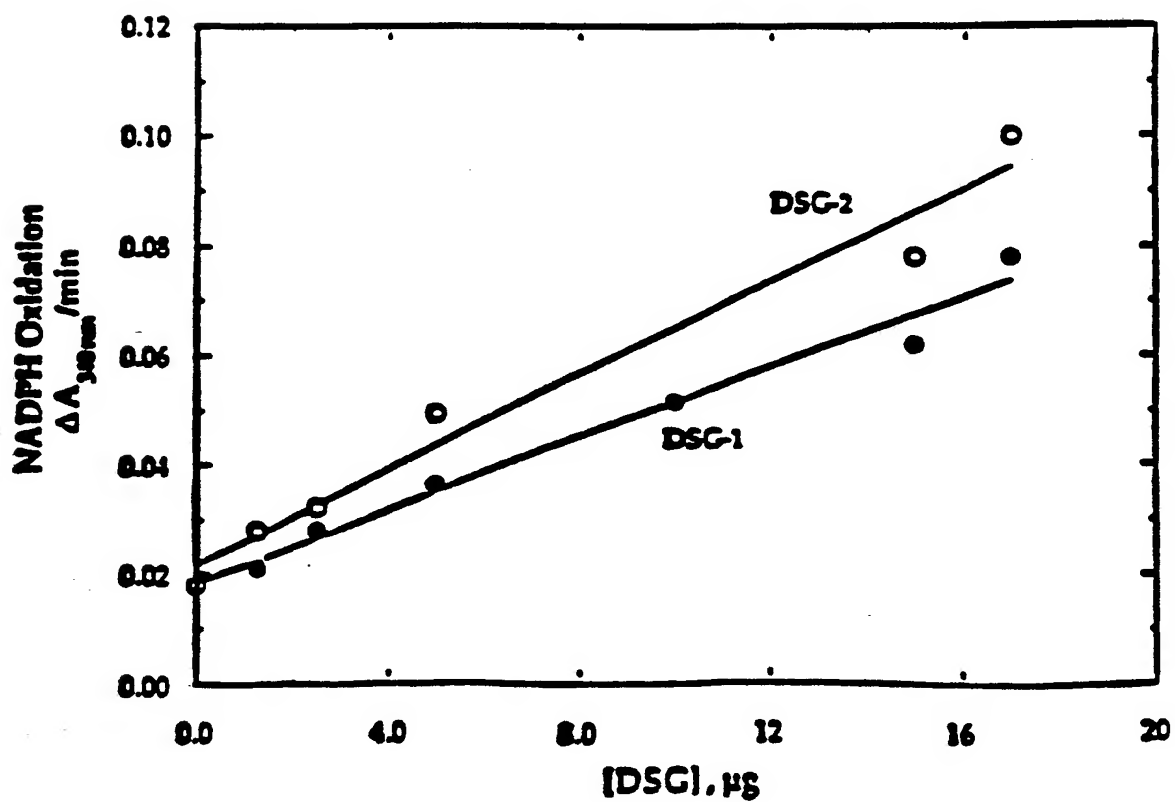


FIG. 1

FIG. 2



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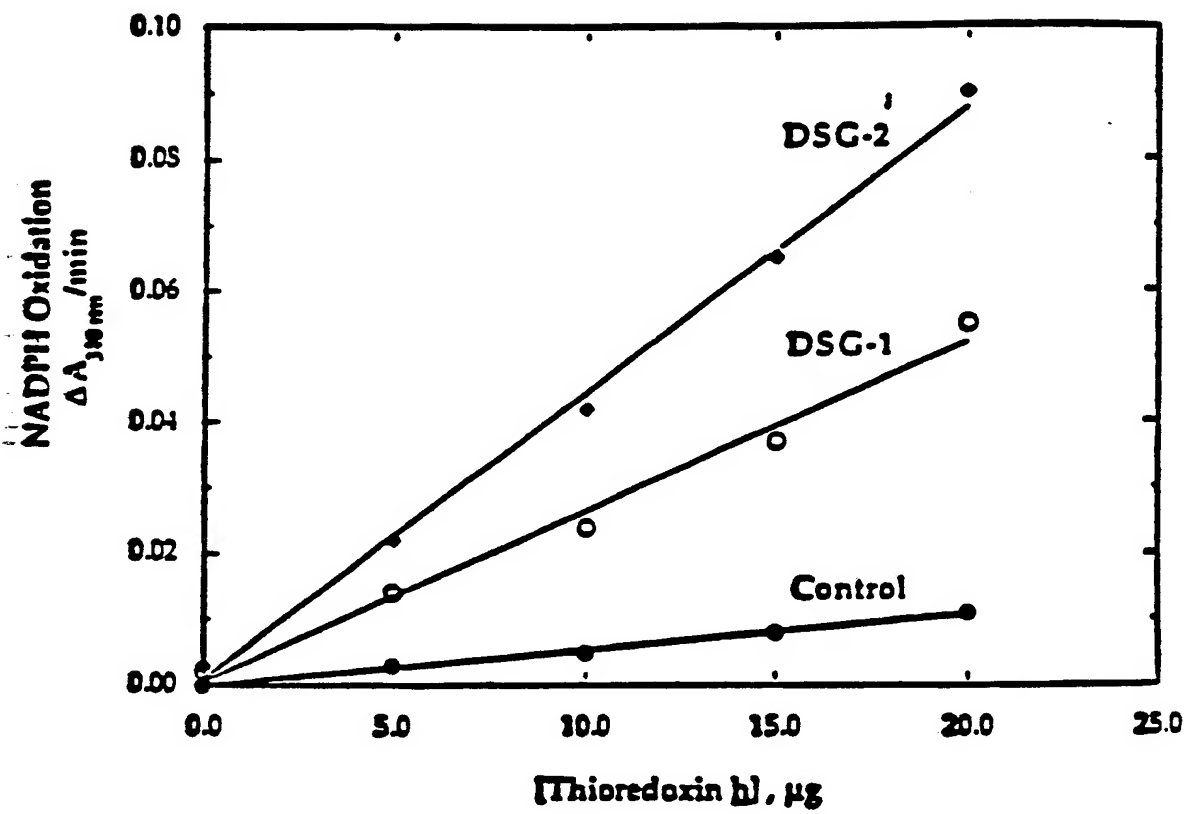


FIG. 3

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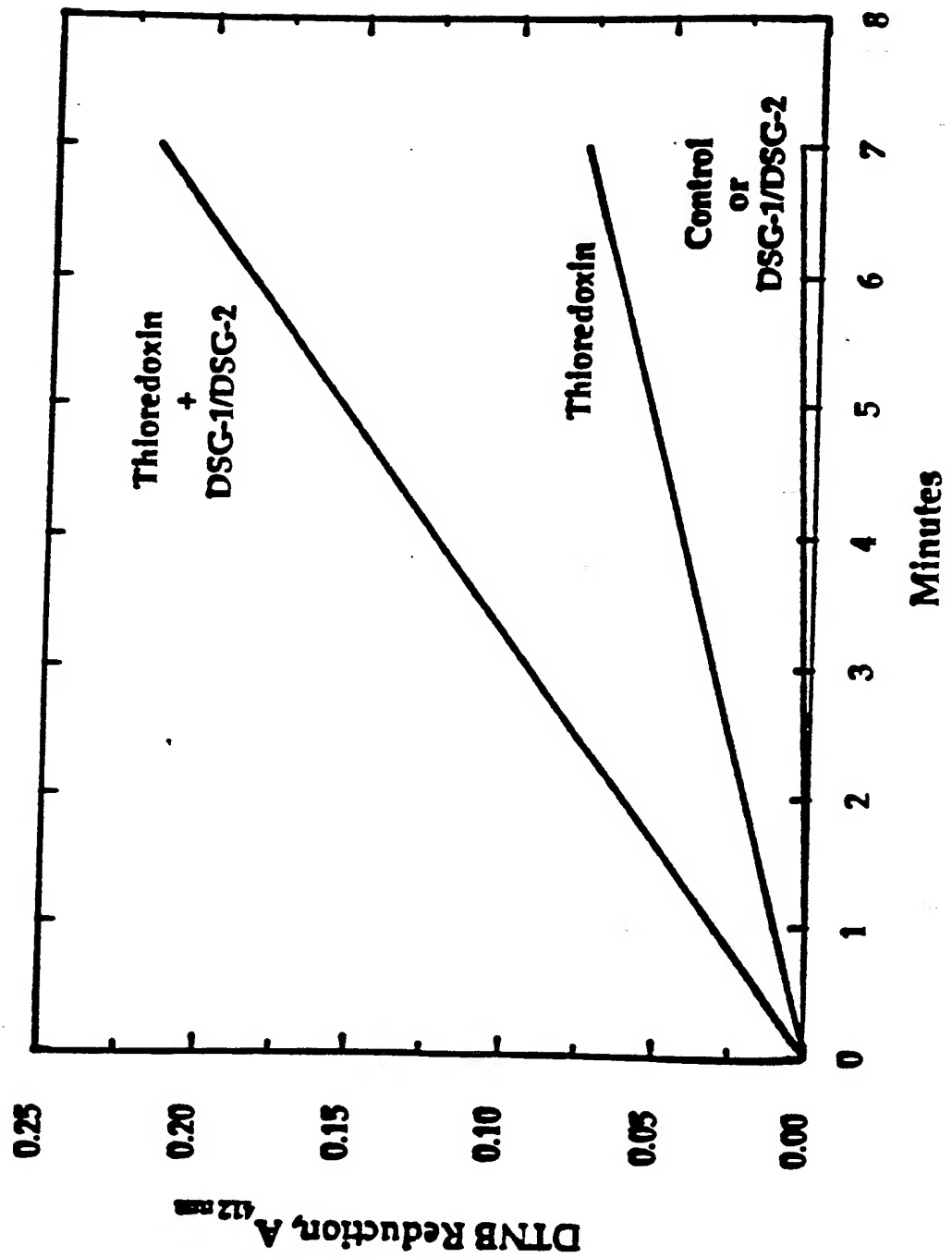


FIG. 4

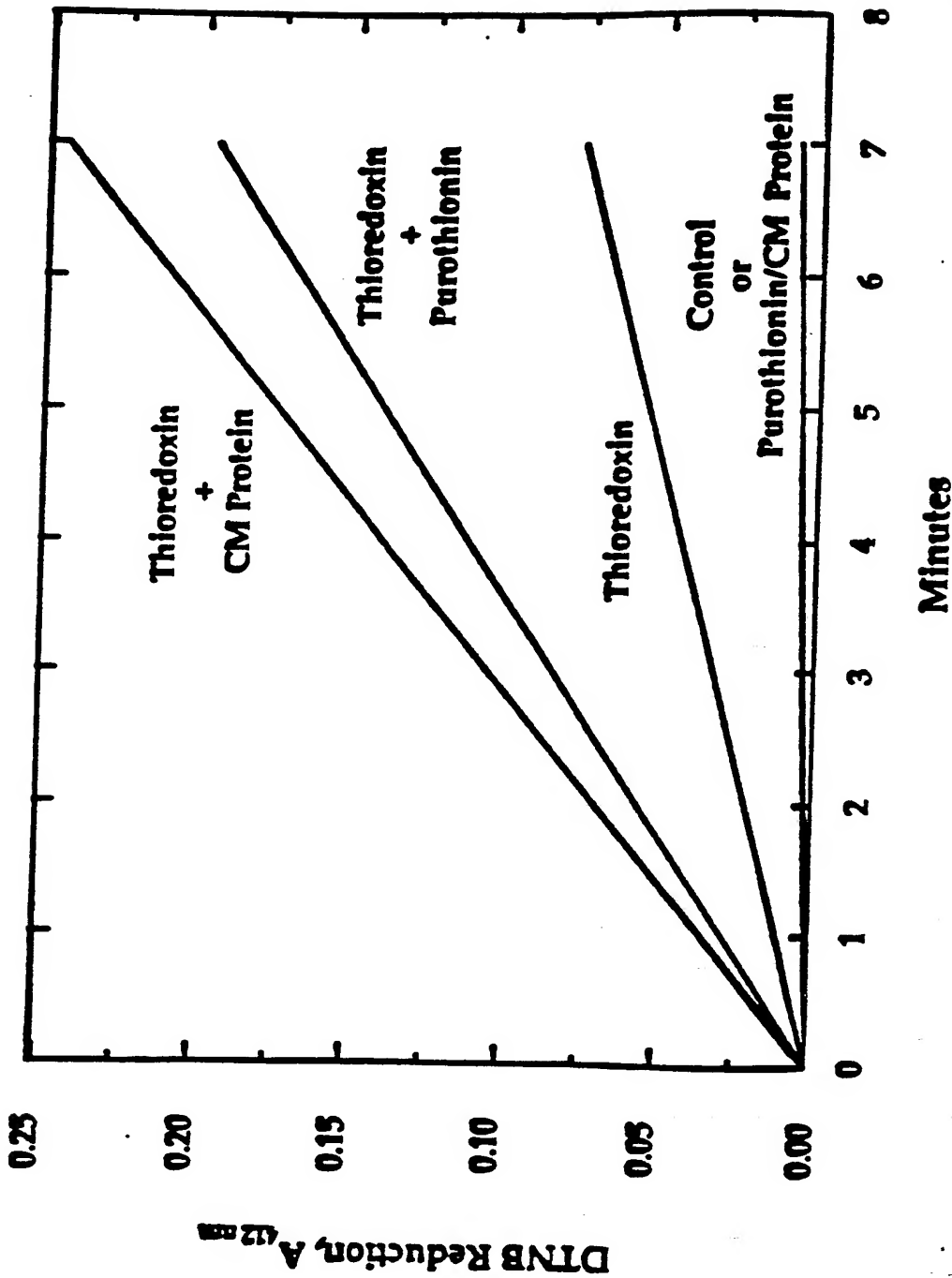
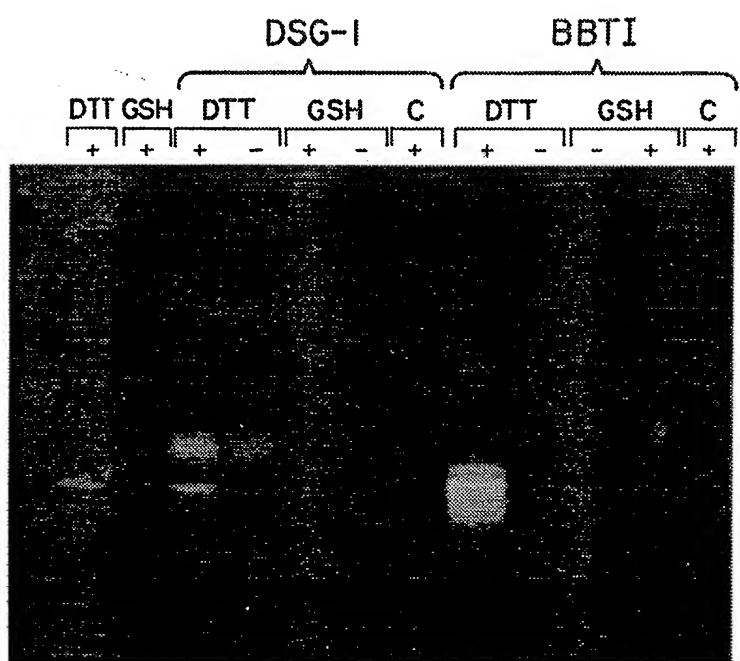


FIG. 5

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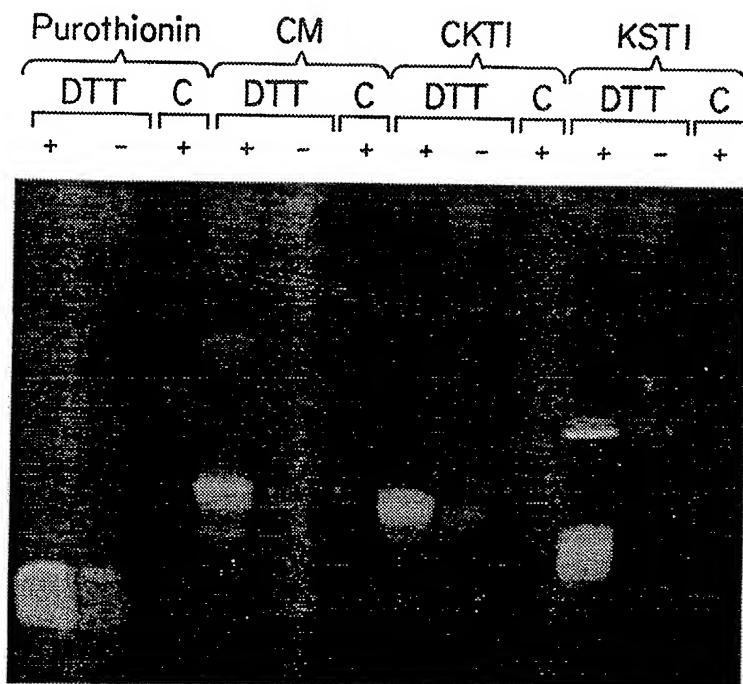
+ and - refer to thioredoxin (*E. coli*)

C = Control (no reductant)

◊ = Thioredoxin

FIG. 6

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+ and - refer to thioredoxin (*E. coli*)

C = Control (no reductant)

◇ = Thioredoxin

CM = Soluble bread wheat sulfur rich protein

CKTI = Corn kernel trypsin inhibitor

KSTI = Kunitz soybean trypsin inhibitor

FIG. 7

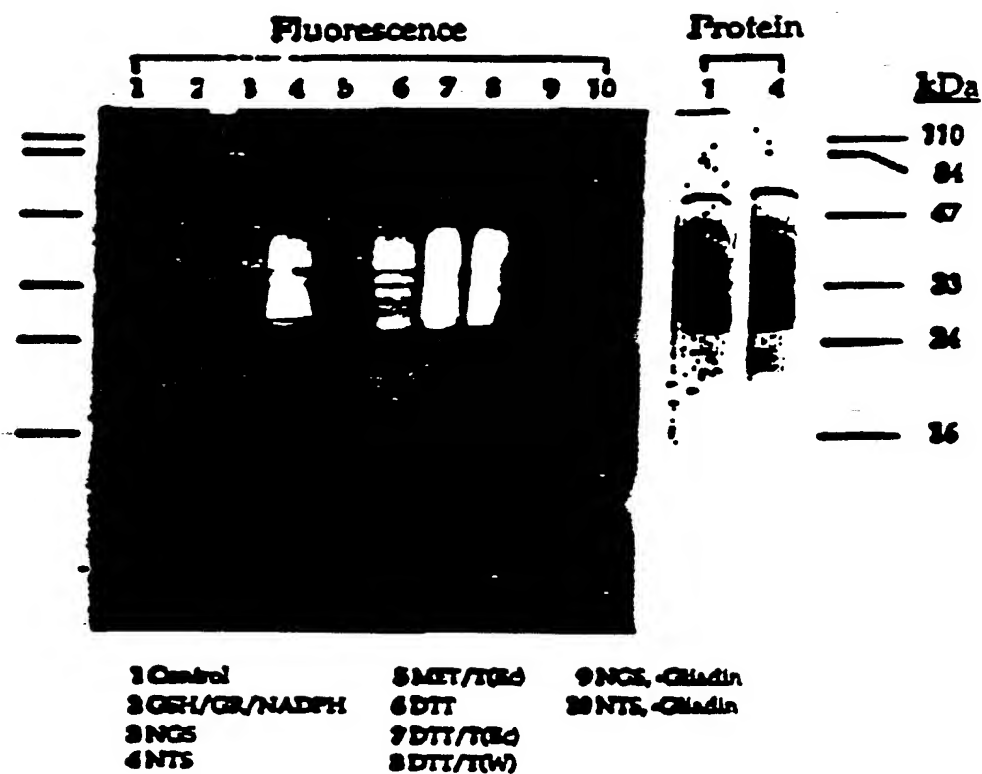
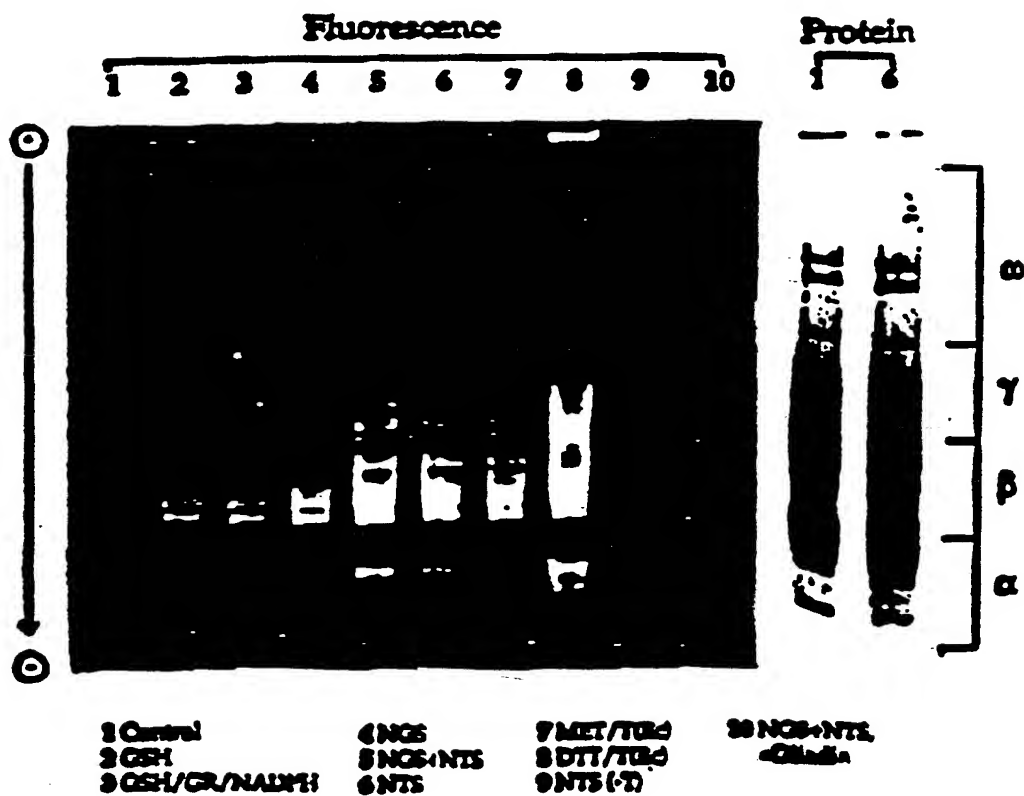


FIG. 8

FIG. 9



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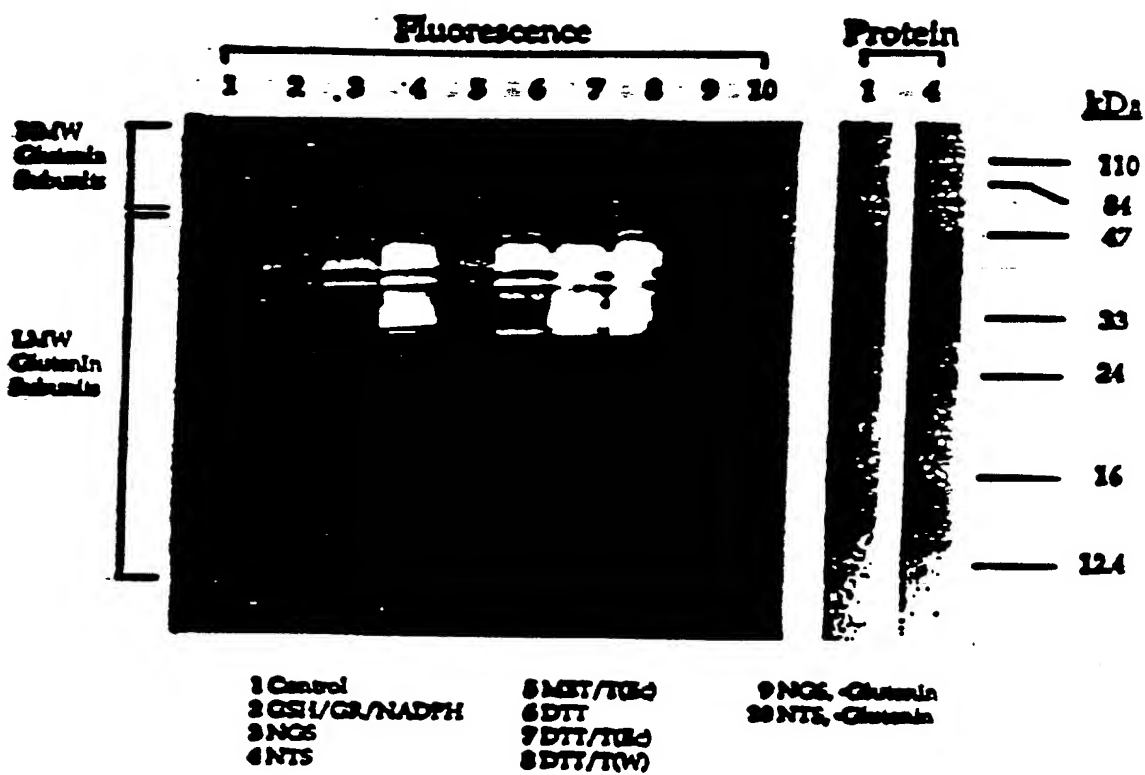
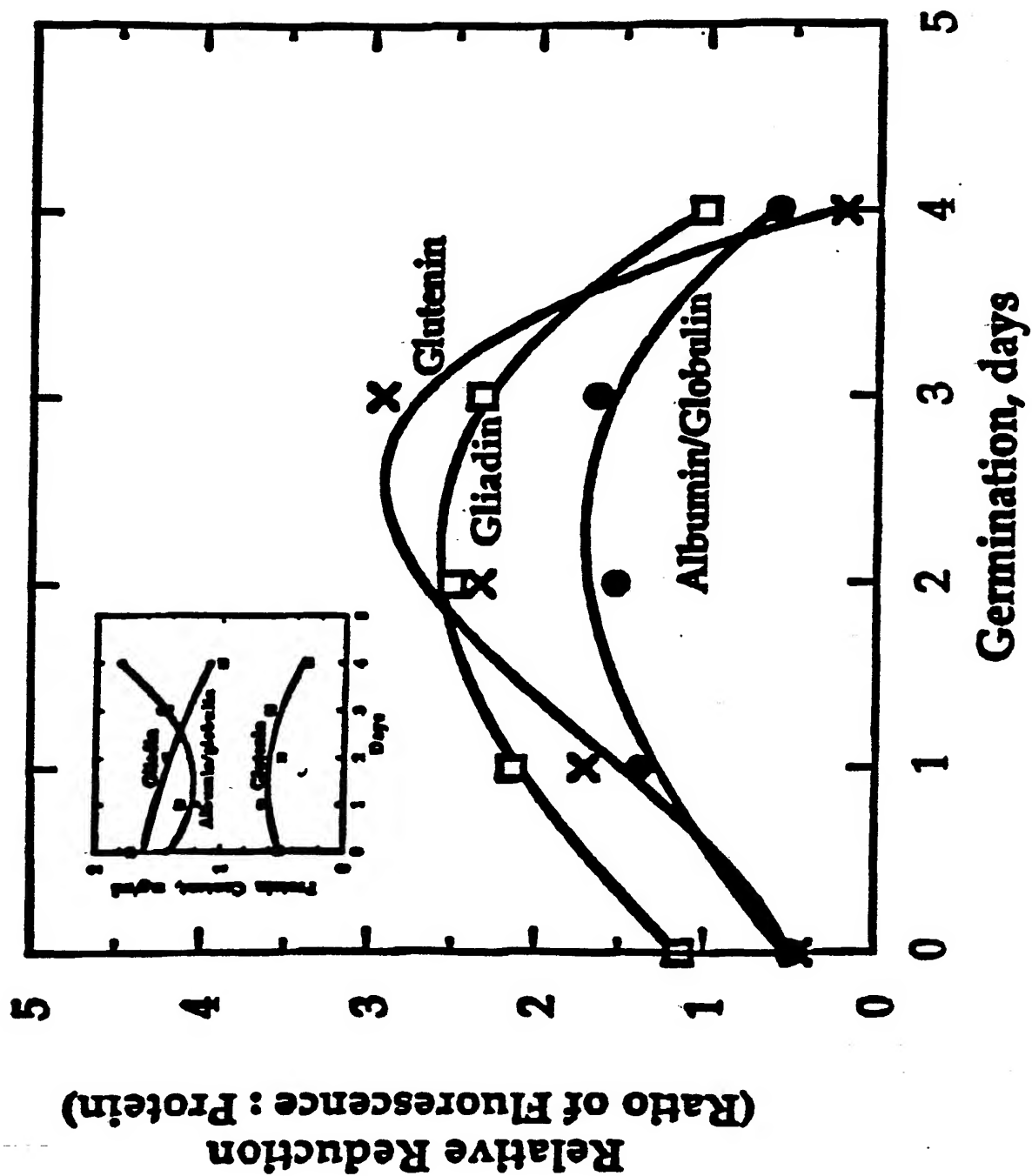


FIG. 10

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FIG. 11



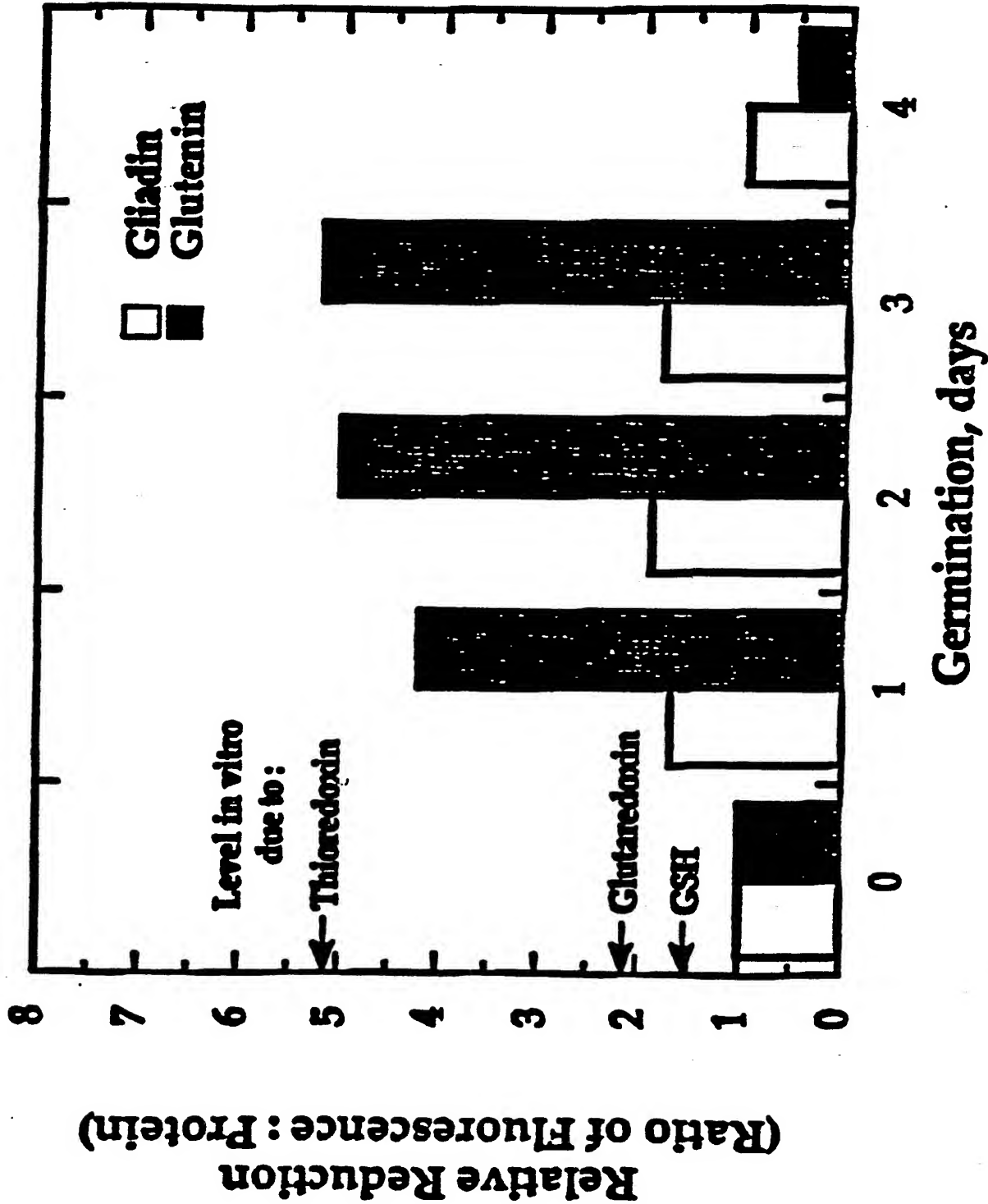


FIG. 12

# Proposed Role of Thioredoxin In Forming Protein Network for Bread and Pasta

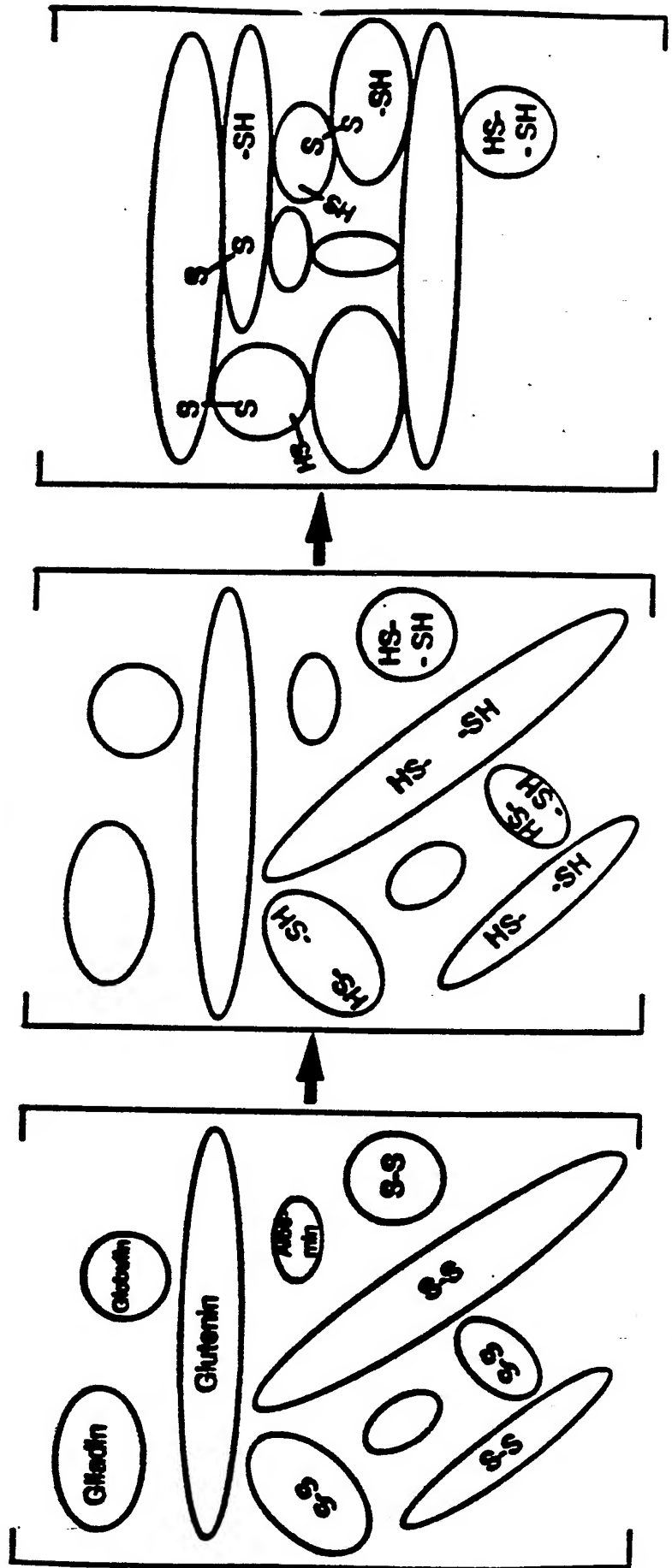
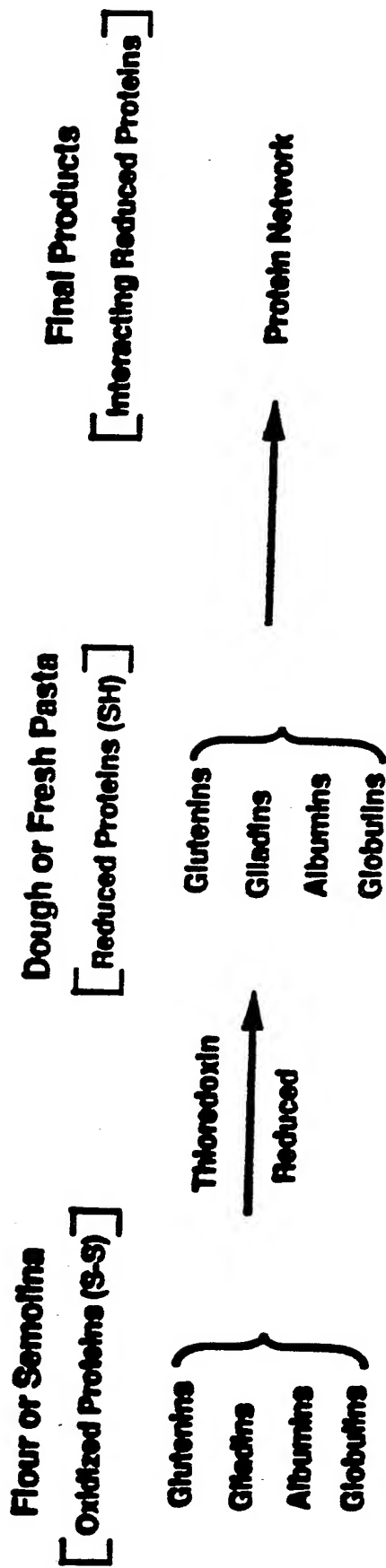


FIG. 13

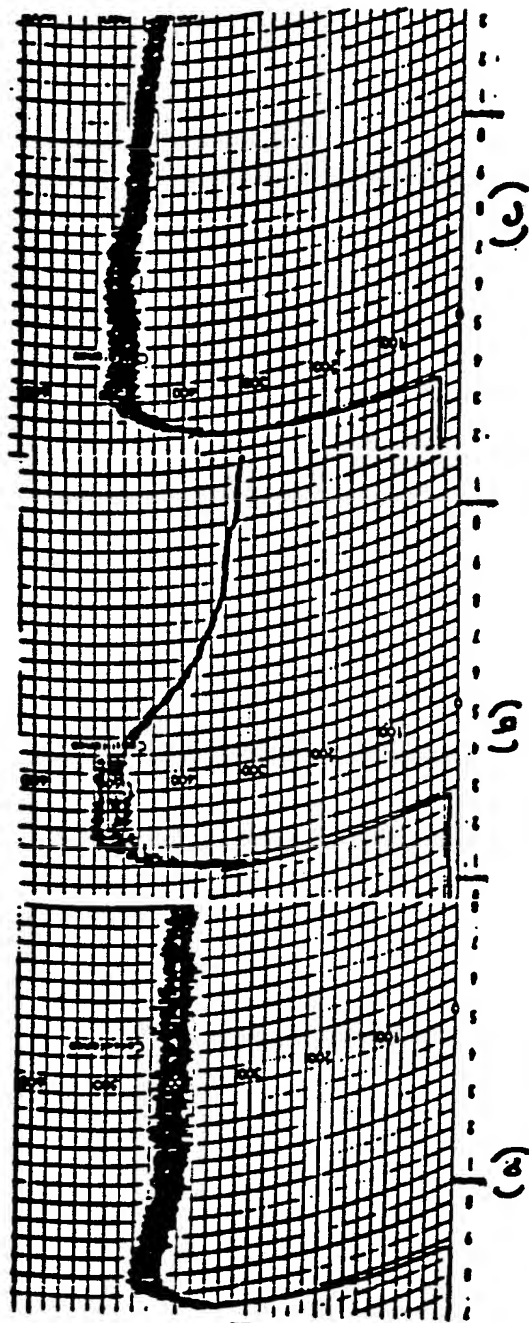


FIG. 14

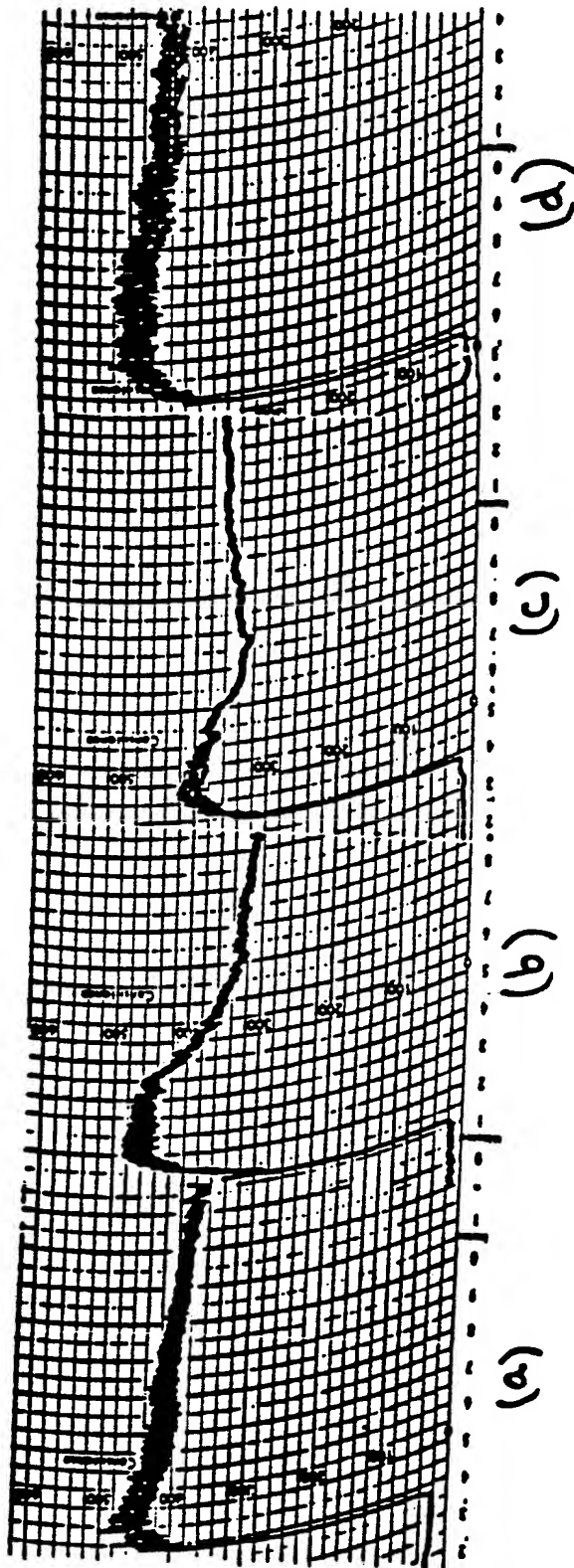


FIG. 15

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WHEAT CULTIVAR: APOLLO

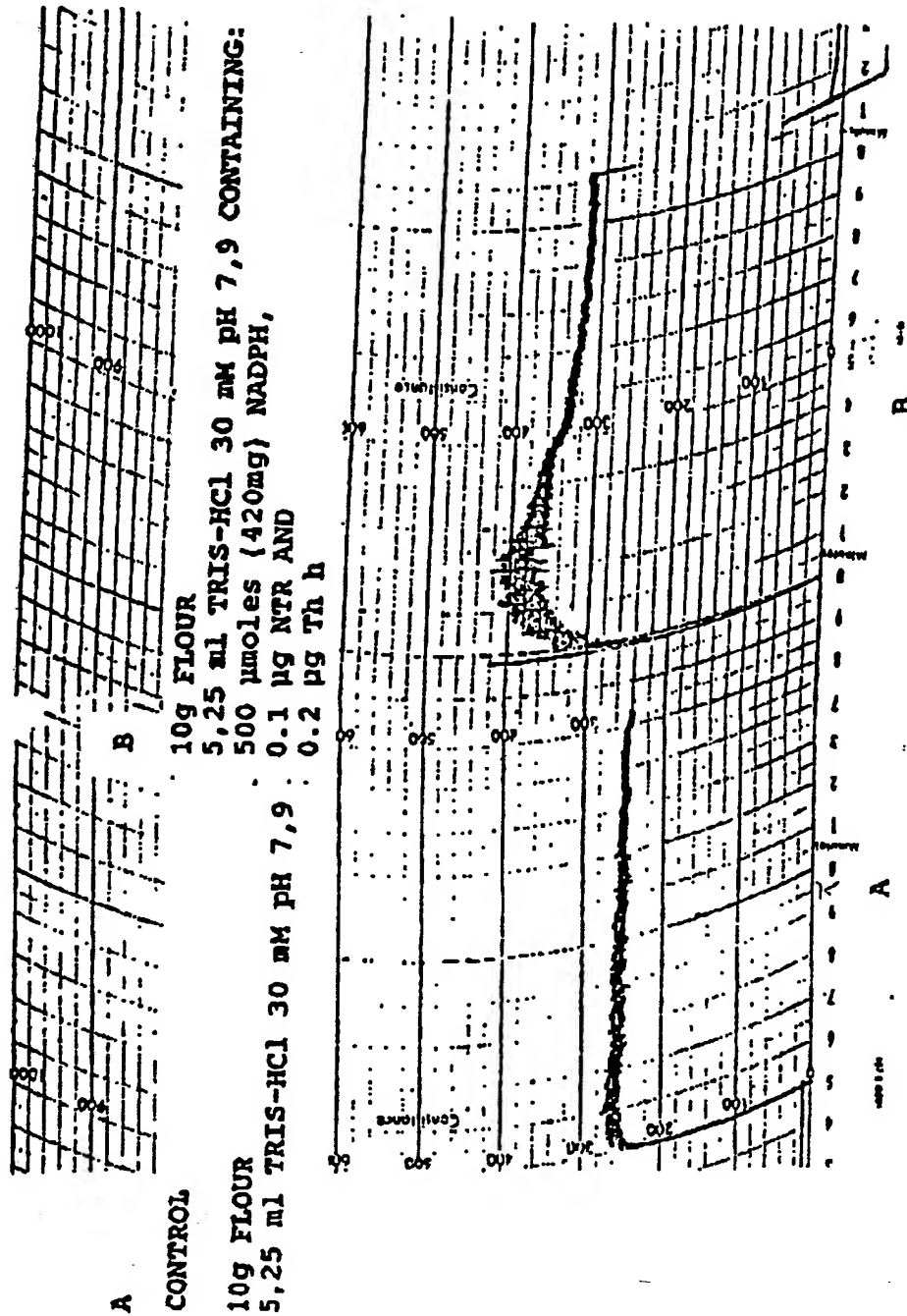
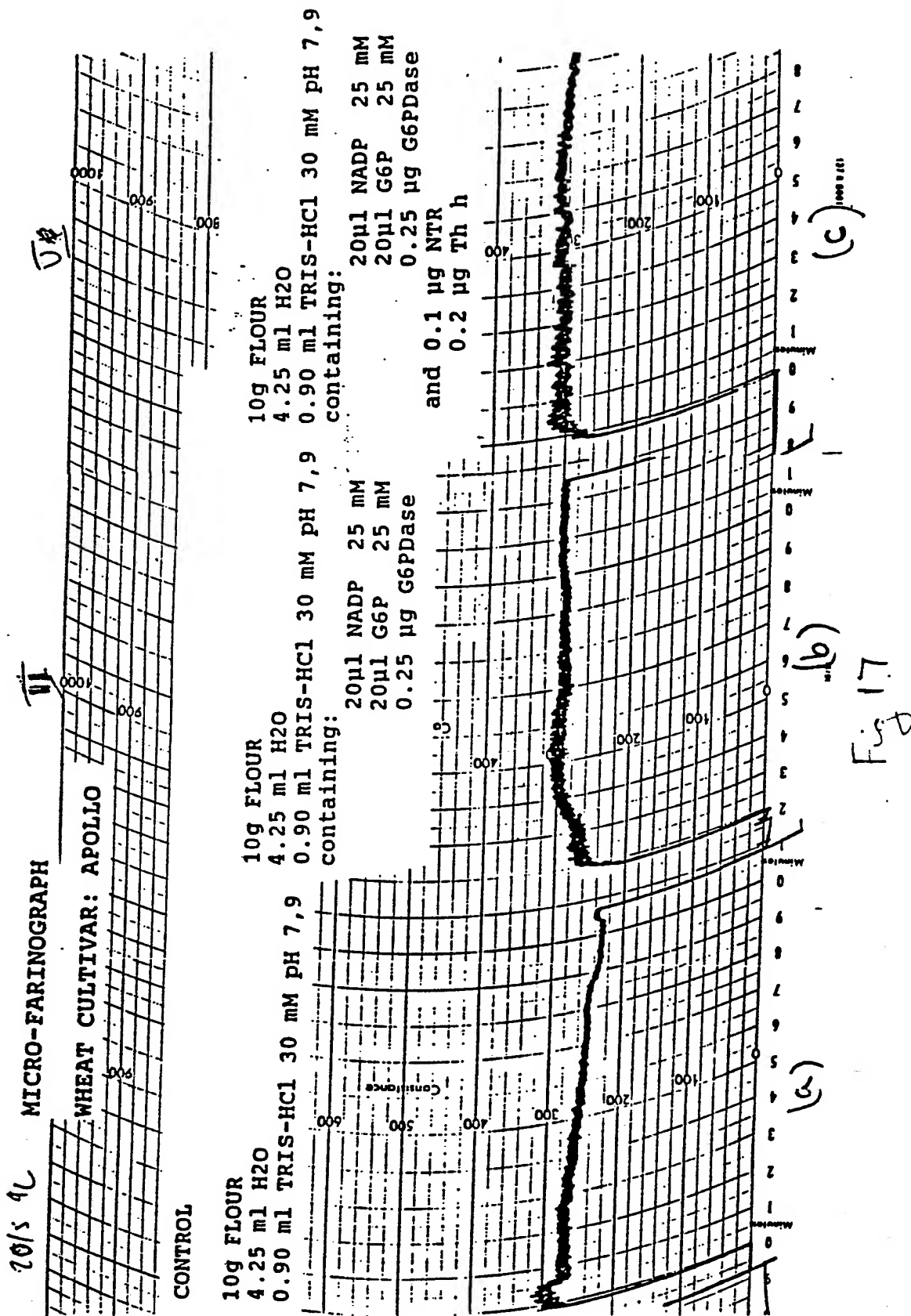


Fig.16

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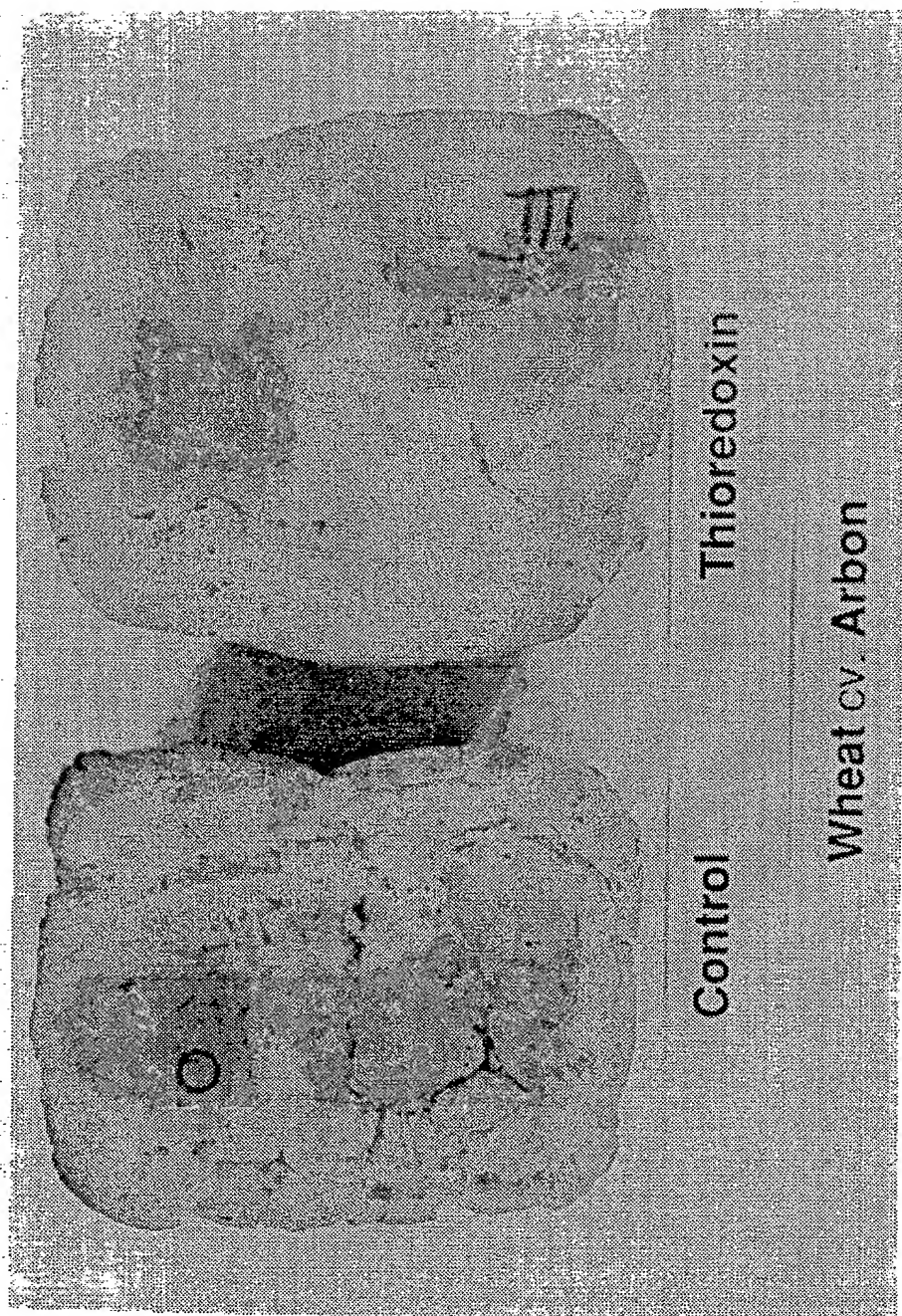
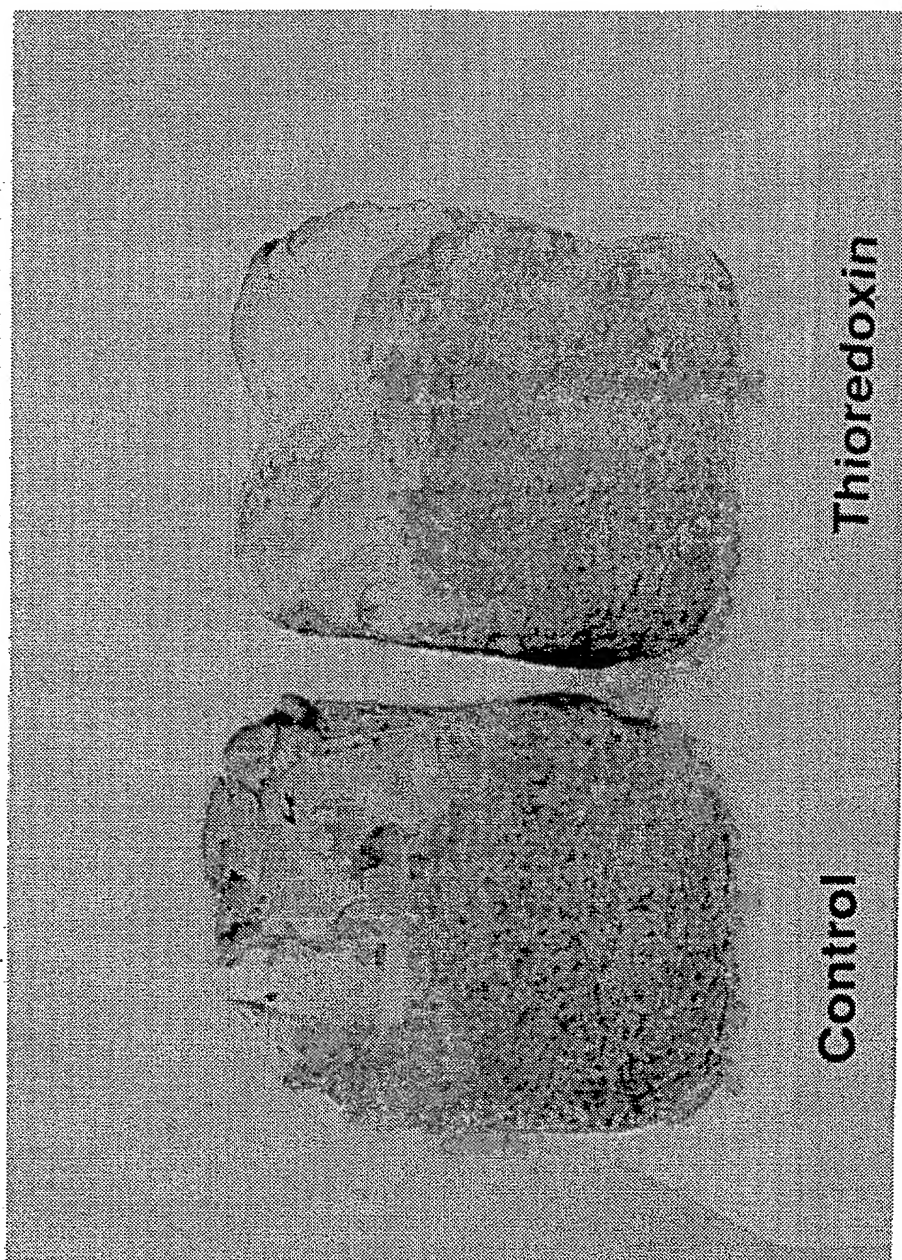


FIG. 18

**FIG. 19**

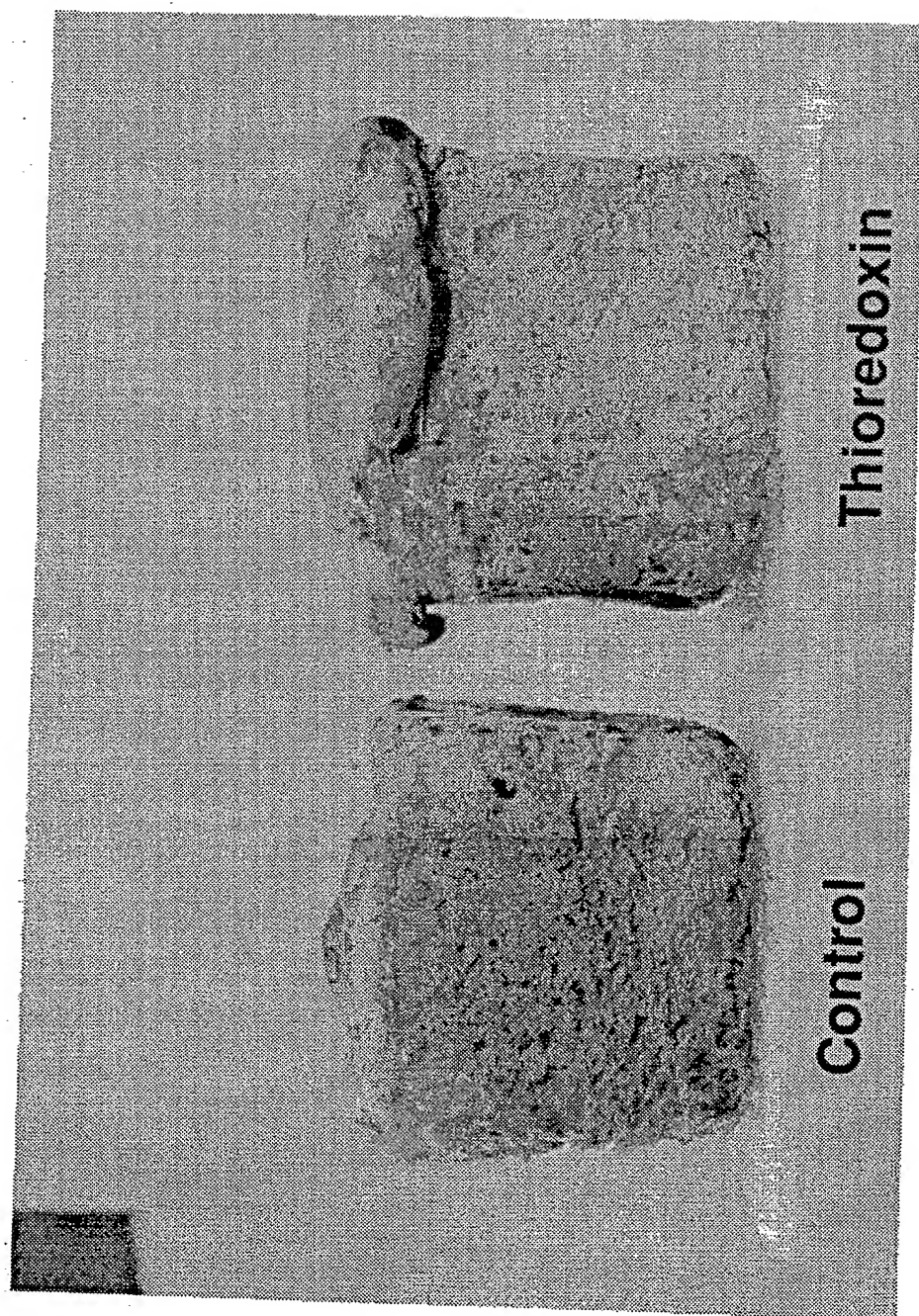
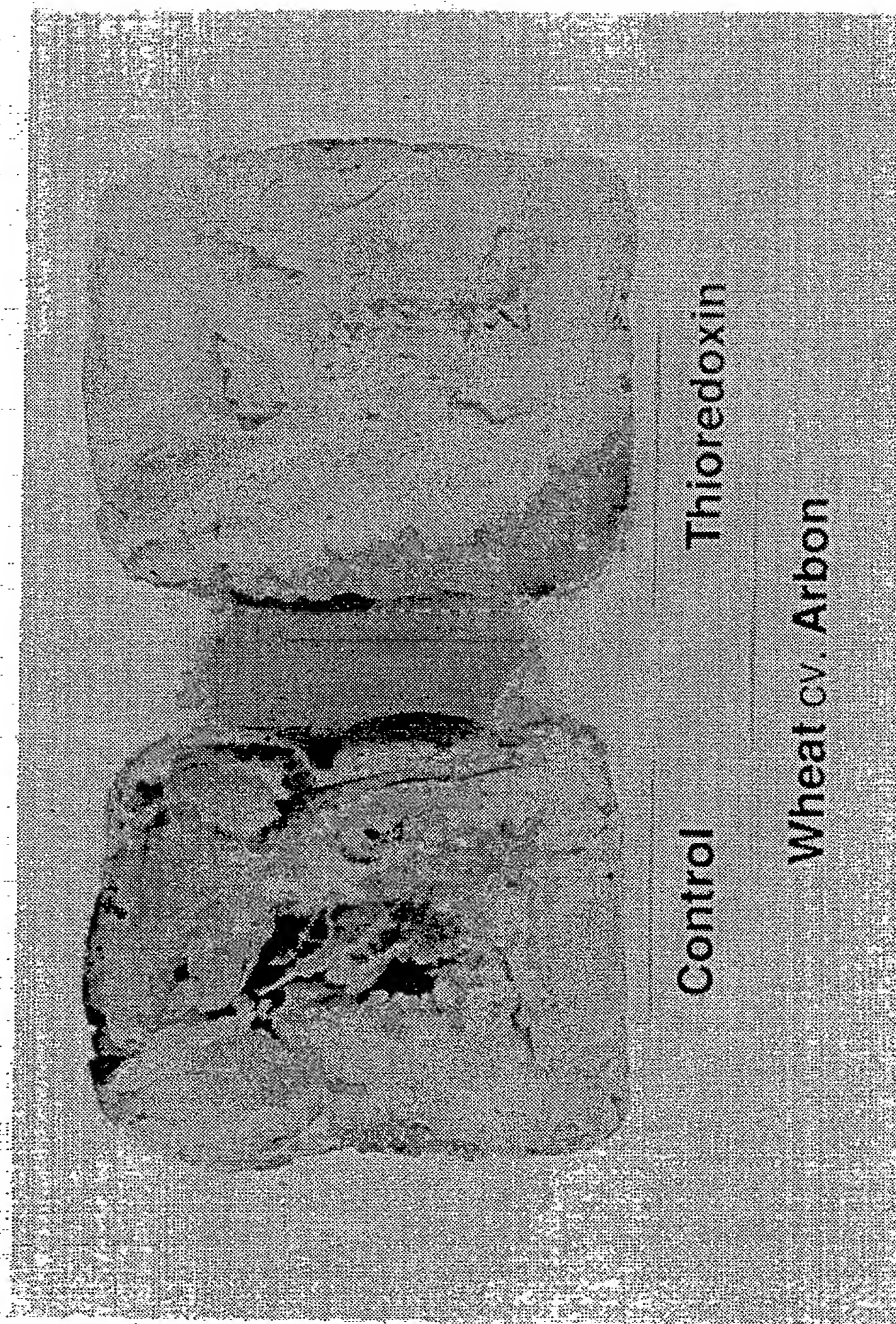


FIG. 20

**FIG. 21**

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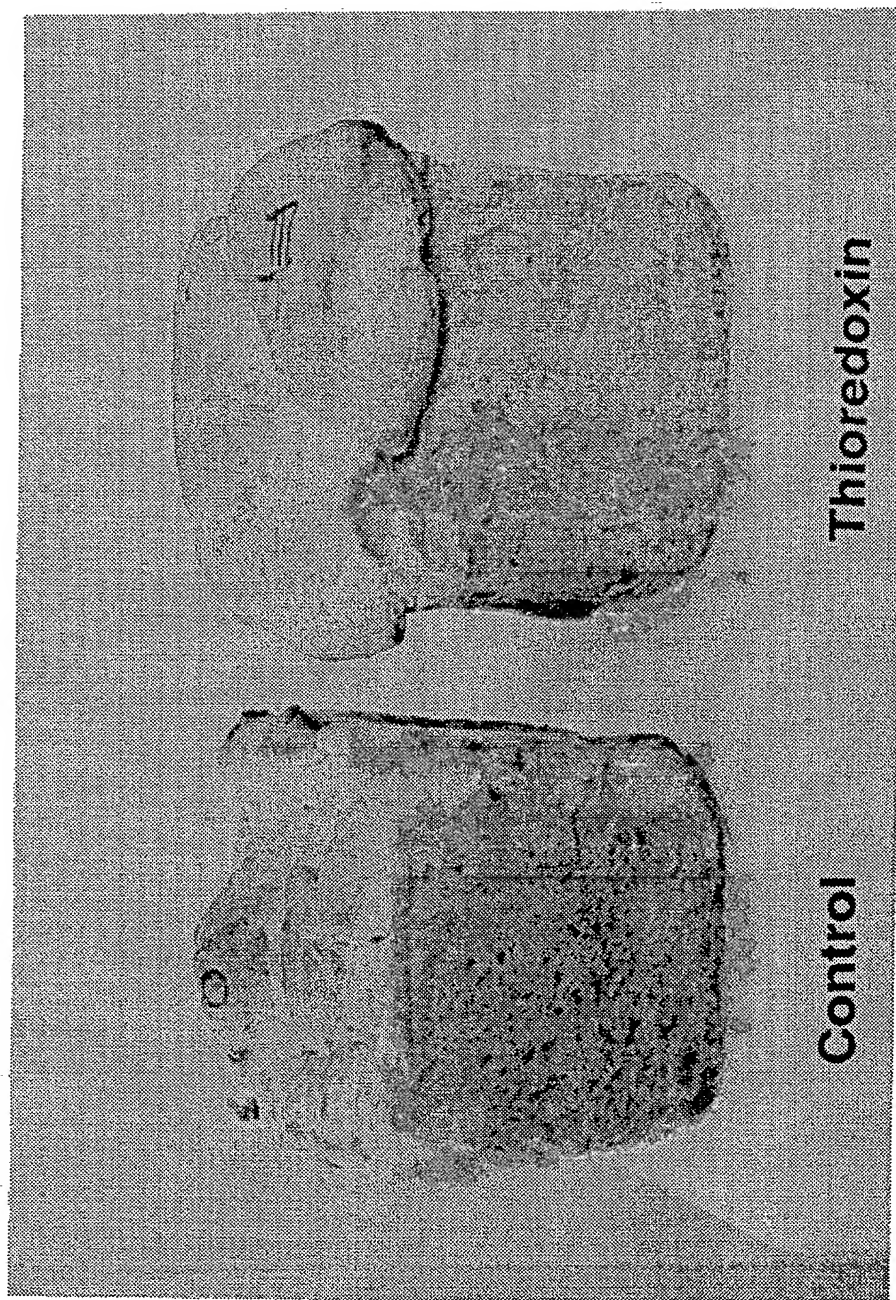
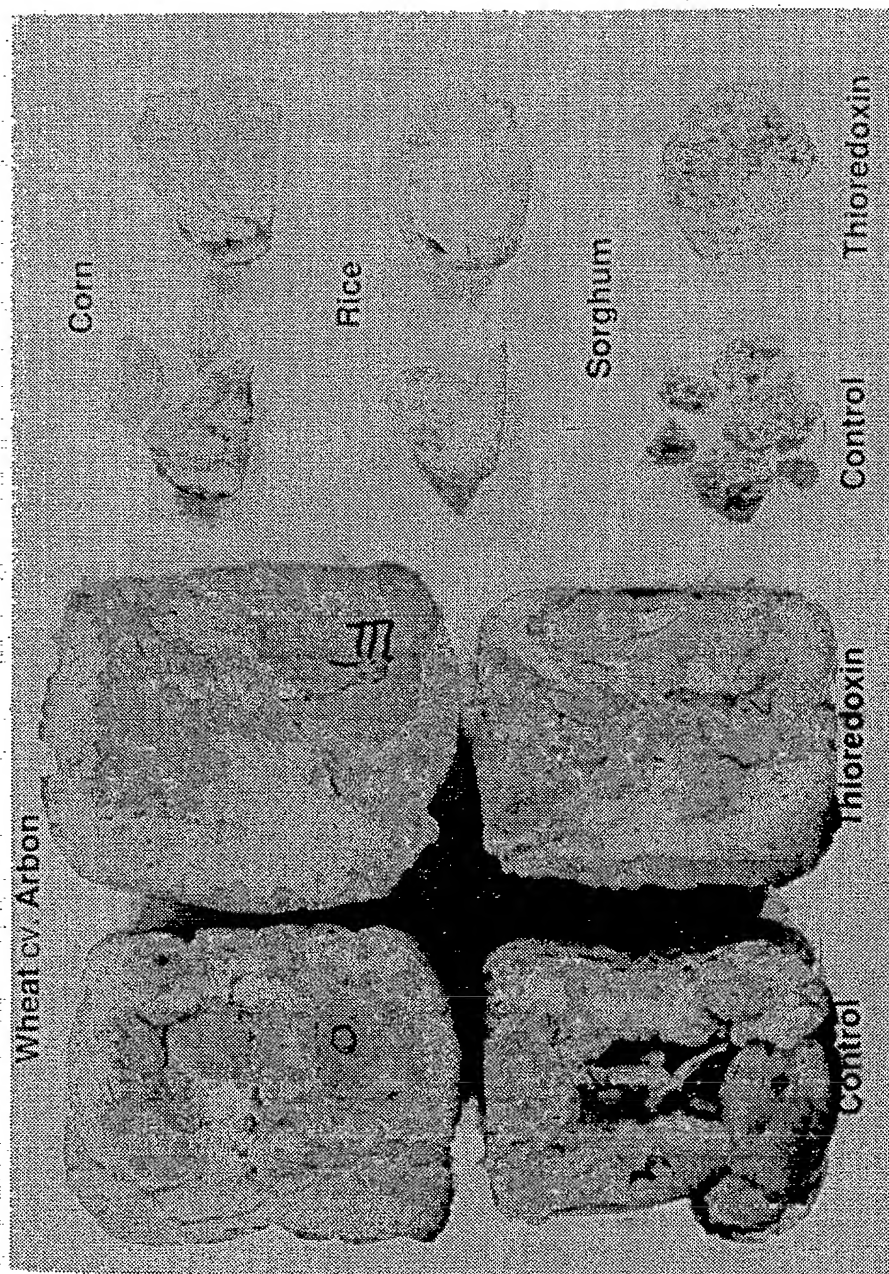


FIG. 22

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B

FIG. 23

A

22 / 50

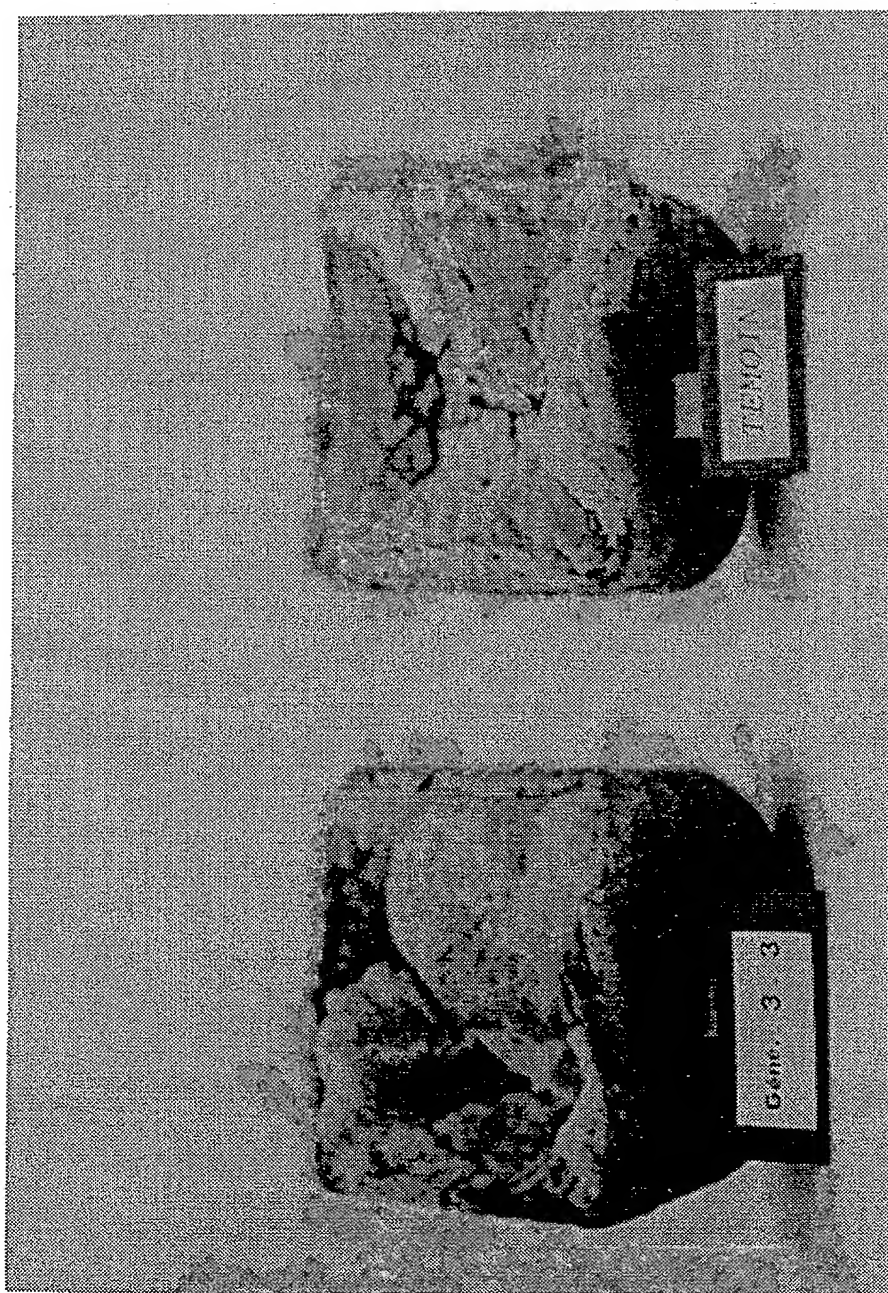
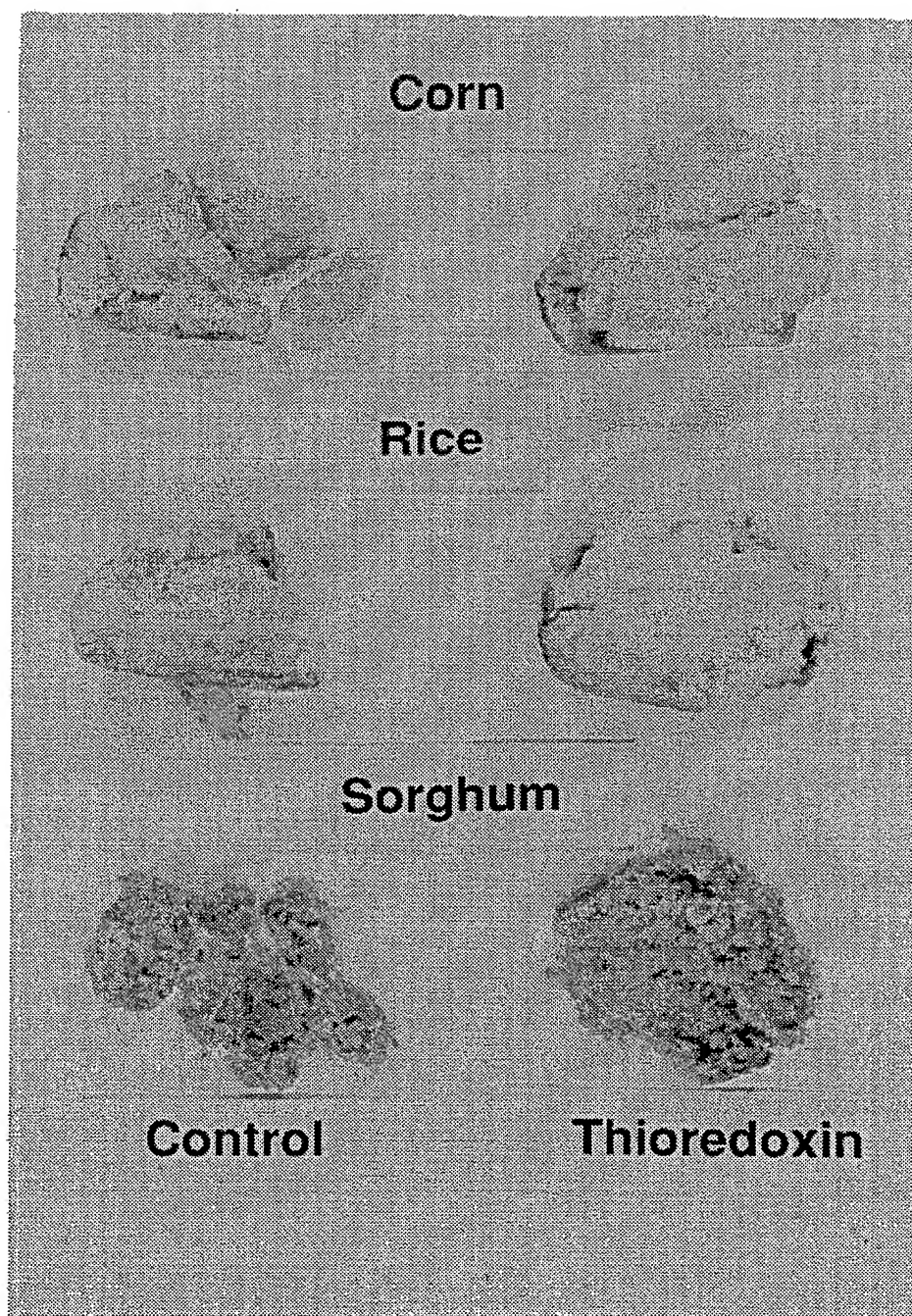


FIG. 24

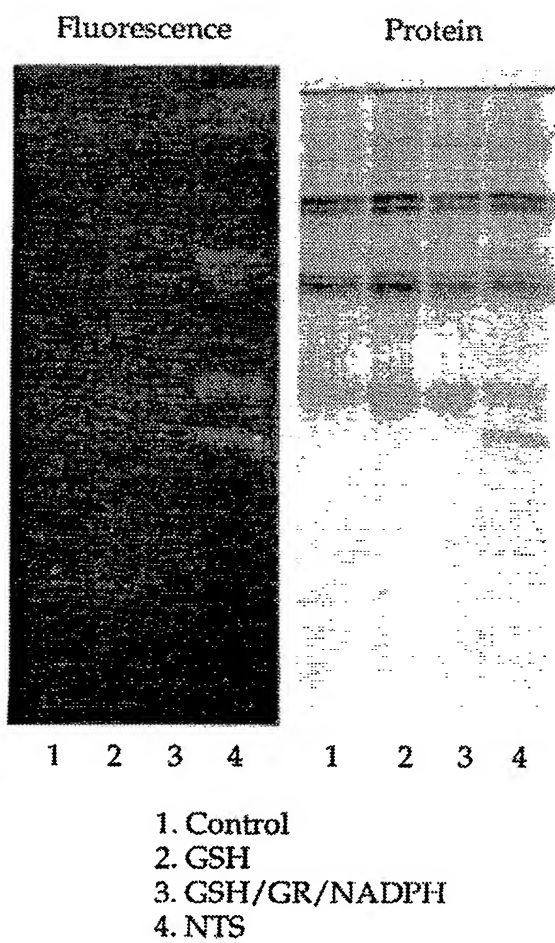
23/50



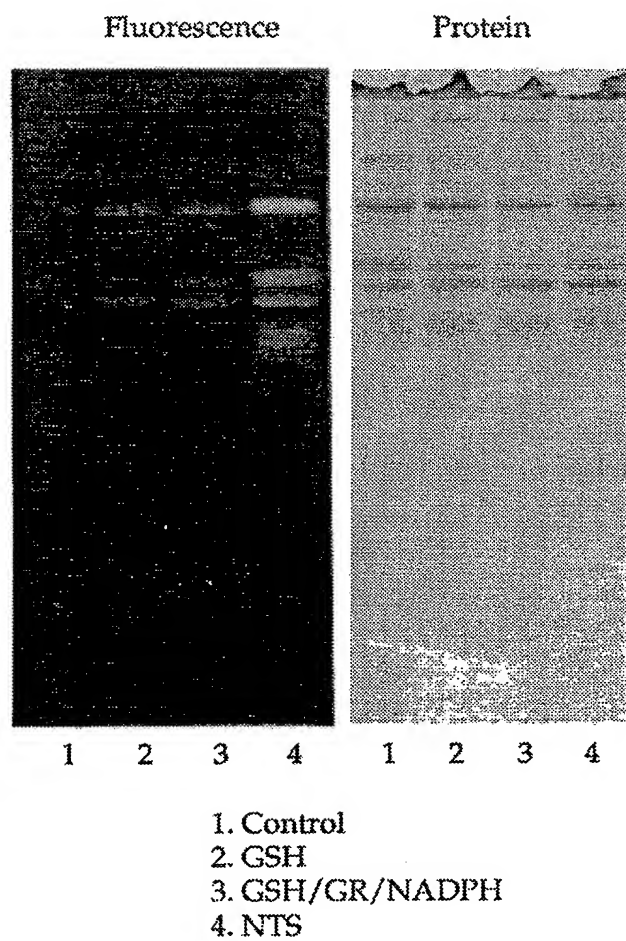
**FIG. 25**

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Thioredoxin-linked Reduction of Myristate-Extracted Proteins  
from Oat Flour

**FIG. 26**

Thioredoxin-linked Reduction of Myristate-Extracted Proteins  
from Triticale Flour



**FIG. 27**

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Thioredoxin-linked Reduction of Myristate-Extracted Proteins  
from Rye Flour

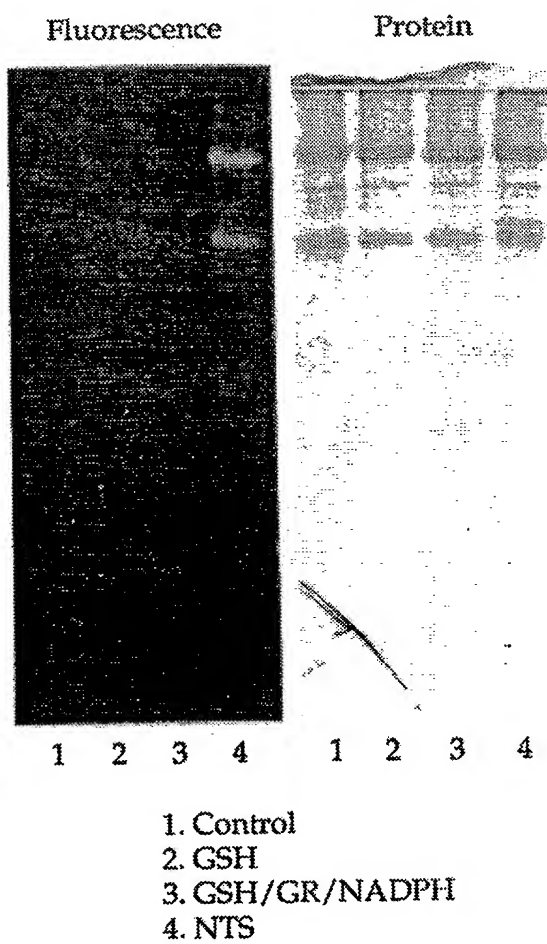


FIG. 28

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Thioredoxin-linked Reduction of Myristate-Extracted Proteins  
from Barley Flour

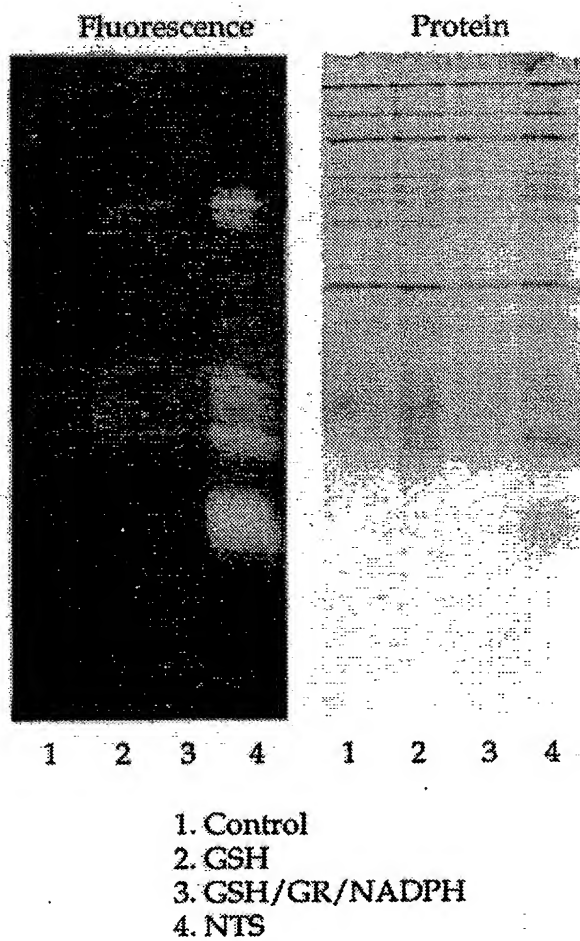
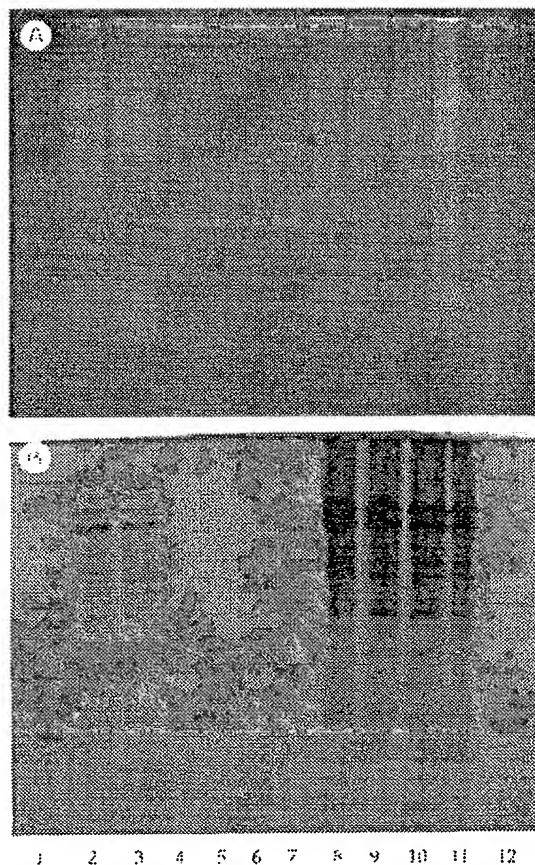


FIG. 29

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The reduced-linked Reduction of Buffer-, Ethanol- and Myristate- Extracted Proteins from TcII: (A) Fluorescence (B) Protein



- |                              |                                |
|------------------------------|--------------------------------|
| 1. MW markers                | 8. Myristate-Extracted Control |
| 2. Buffer-Extracted Control  | 9. " + GSH                     |
| 3. " + NIS                   | 10. " + GSH/GR/NADPH           |
| 4. Ethanol-Extracted Control | 11. " + NTN                    |
| 5. " + GSH                   | 12. MW markers                 |
| 6. " + GSH/GR/NADPH          |                                |
| 7. " + NTN                   |                                |

FIG. 30

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Effect of NTS vs Glutathione Reductase (GR) on  
Reduction Status of Myristate-Extracted Proteins from  
(A) Corn, (B) Sorghum and (C) Rice

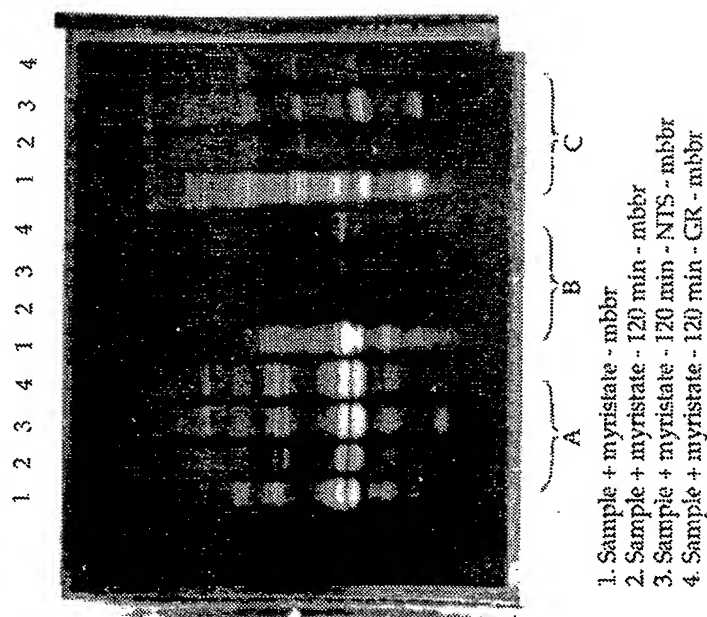
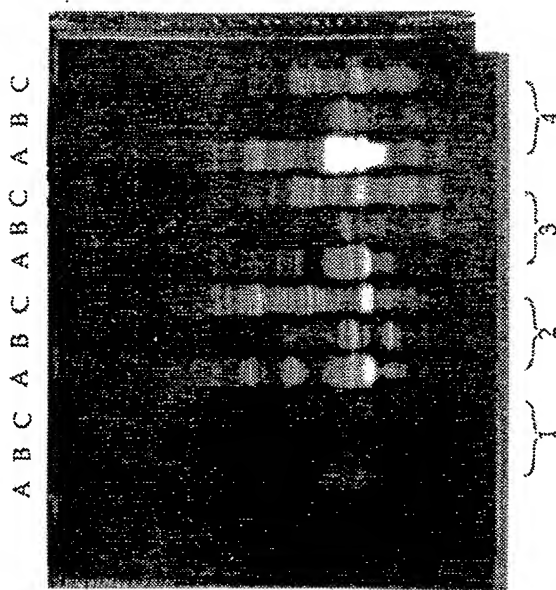


FIG. 31

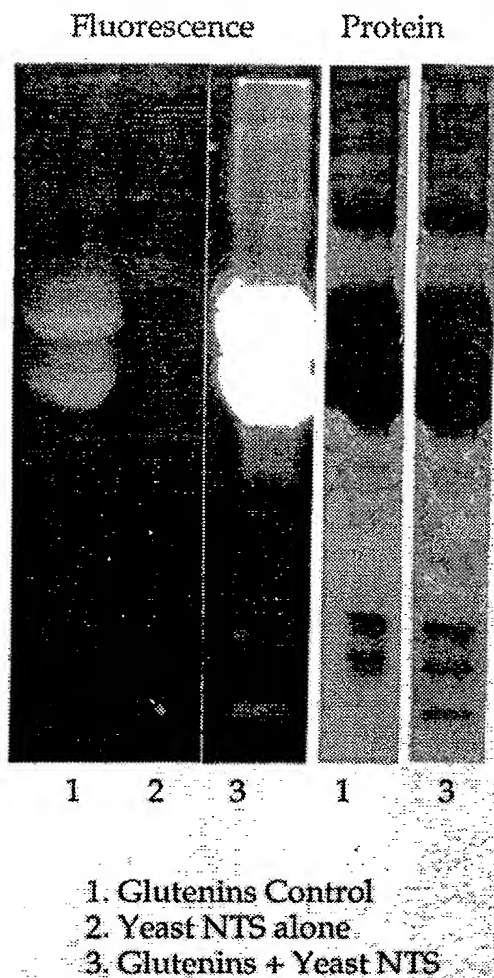
*In vitro* Reduction Status and Thioredoxin-linked *in vitro*  
Reduction of Myristate-Extracted Proteins from (A) Corn,  
(B) Sorghum and (C) Rice



1. Sample + myristate - 120 min - mbbr
2. Sample + myristate - mbbr
3. Sample + myristate - 120 min - NIS - mbbr
4. Sample + myristate - 120 min - DIT - mbbr

FIG. 32

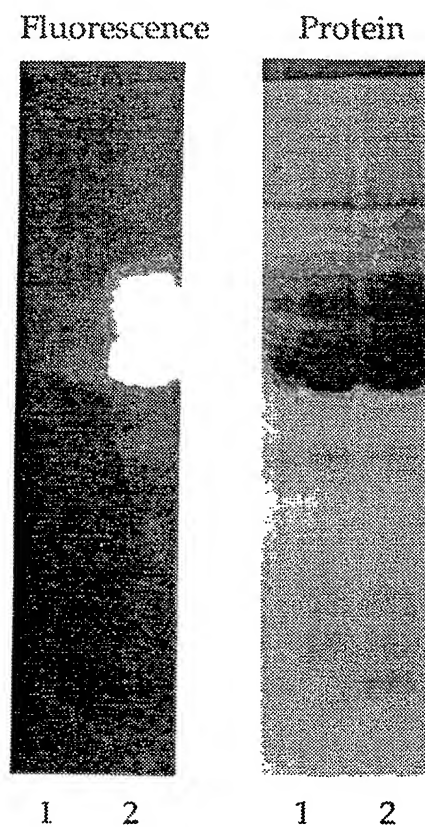
Relative Reduction of Wheat Glutenins  
by Yeast NADP/Thioredoxin System



**FIG. 33**

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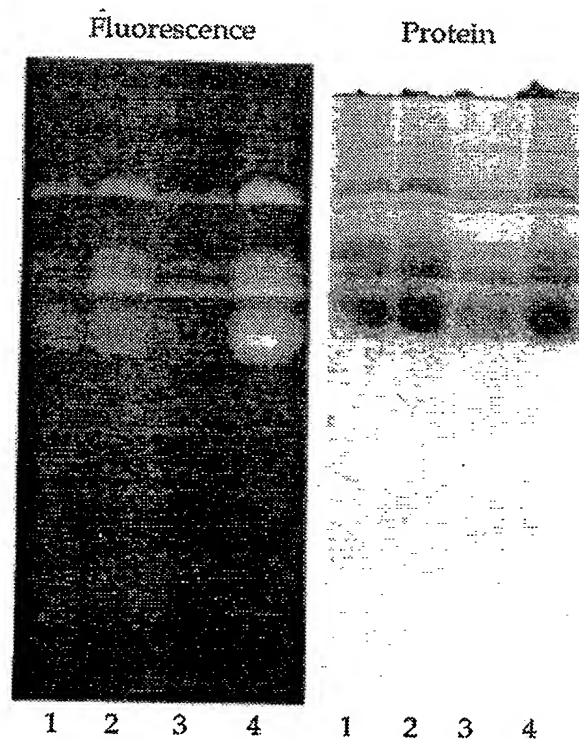
Relative Reduction of Wheat Gliadins  
by Yeast NADP/Thioredoxin System



1. Gliadins Control  
2. Gliadins + Yeast NTS

**FIG. 34**

Thioredoxin-linked Reduction of Ethanol-Extracted Proteins  
from Triticale Flour



1. Control
2. GSH
3. GSH/GR/NADPH
4. NTS

**FIG. 35**

Thioredoxin-linked Reduction of Ethanol-Extracted Proteins  
from Rye Flour

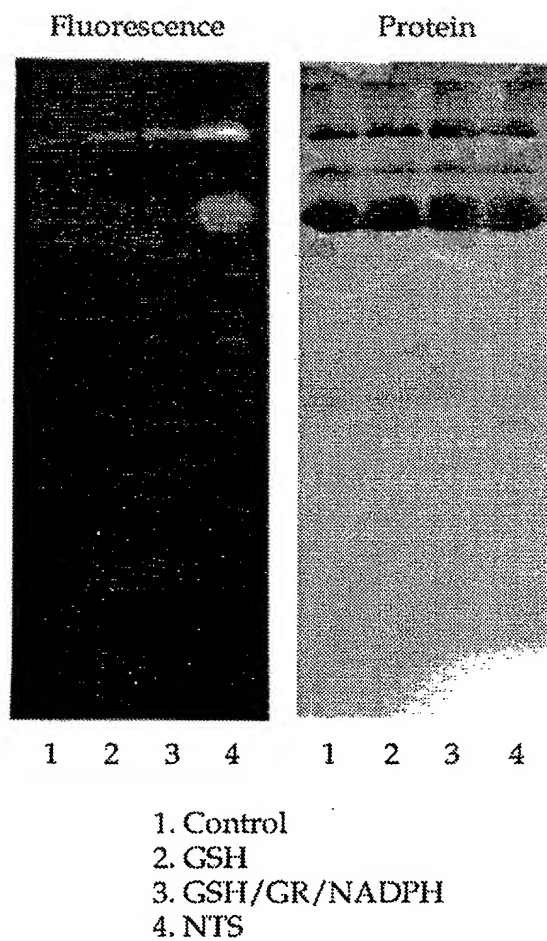


FIG. 36

Thioredoxin-linked Reduction of Ethanol-Extracted Proteins  
from Oat Flour

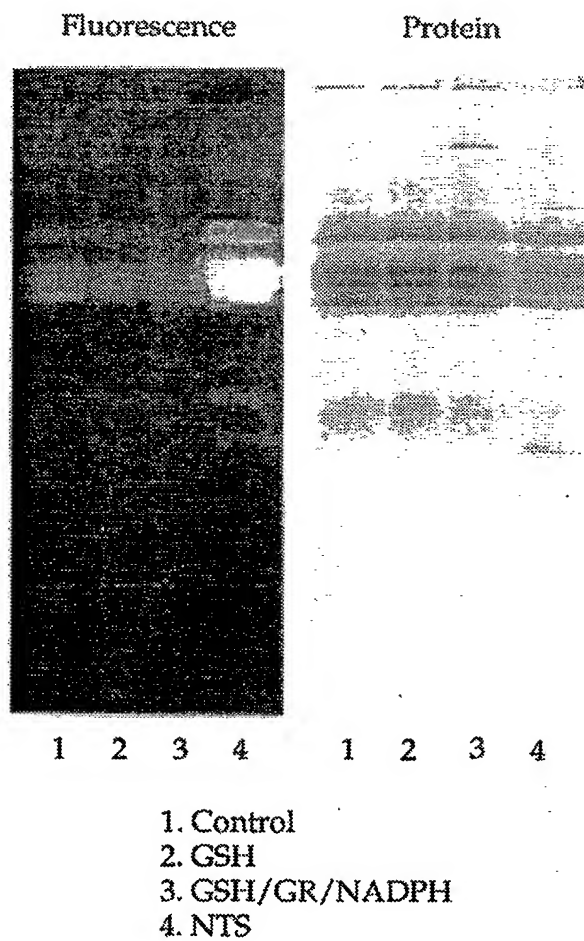
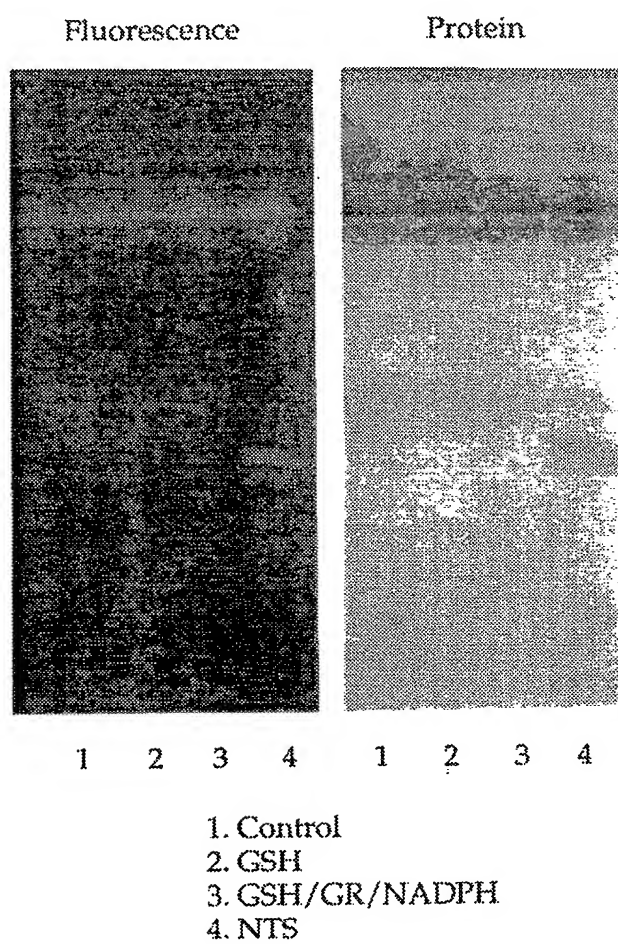


FIG. 37

Thioredoxin-linked Reduction of Ethanol-Extracted Proteins  
from Barley Flour



**FIG. 38**

# Reduction of Castor Seed Matrix and Crystalloid Proteins

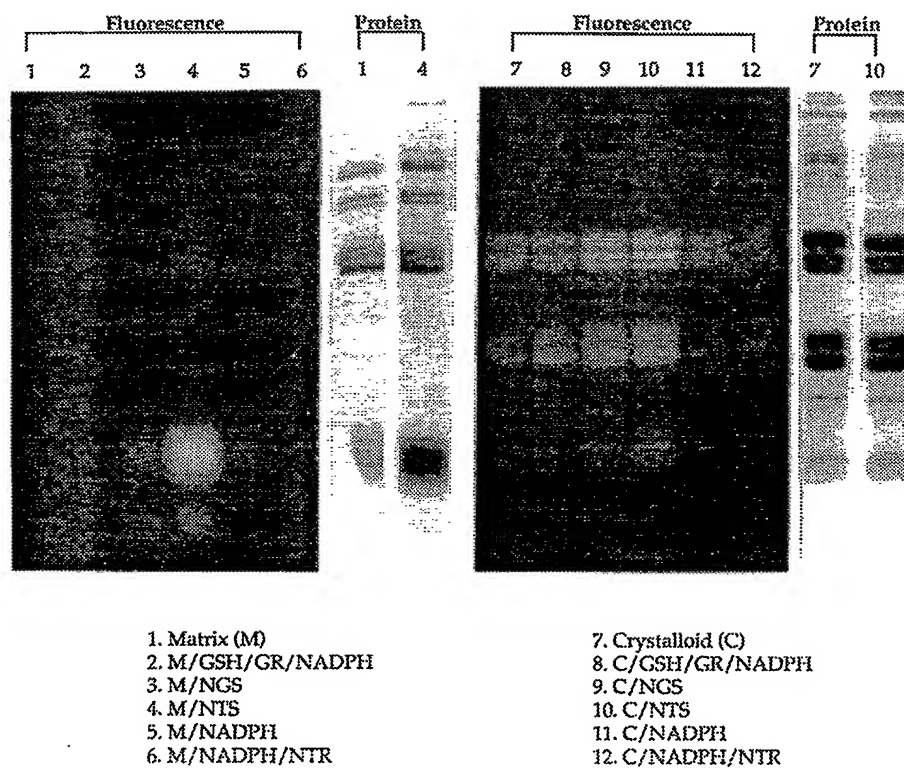
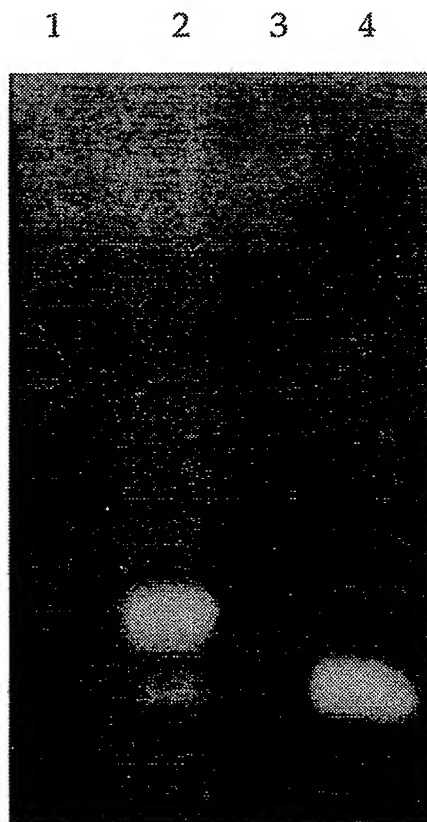


FIG. 39

# Reduction Specificity of 2S Proteins



1. Control
2. Control + NTS
3. Control, 3 min Heat
4. Control + DTT, 3 min Heat

FIG. 40

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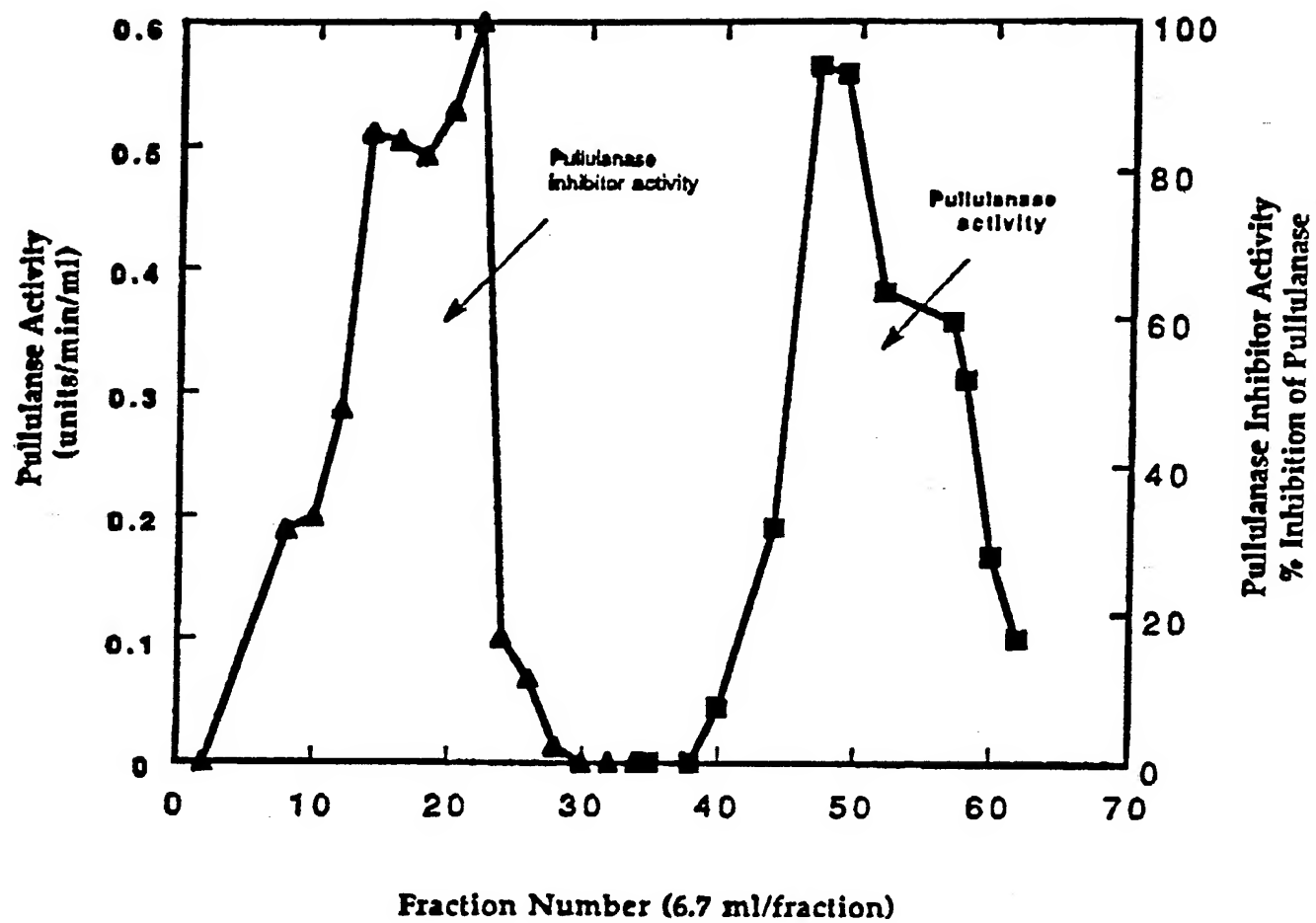


Fig 41

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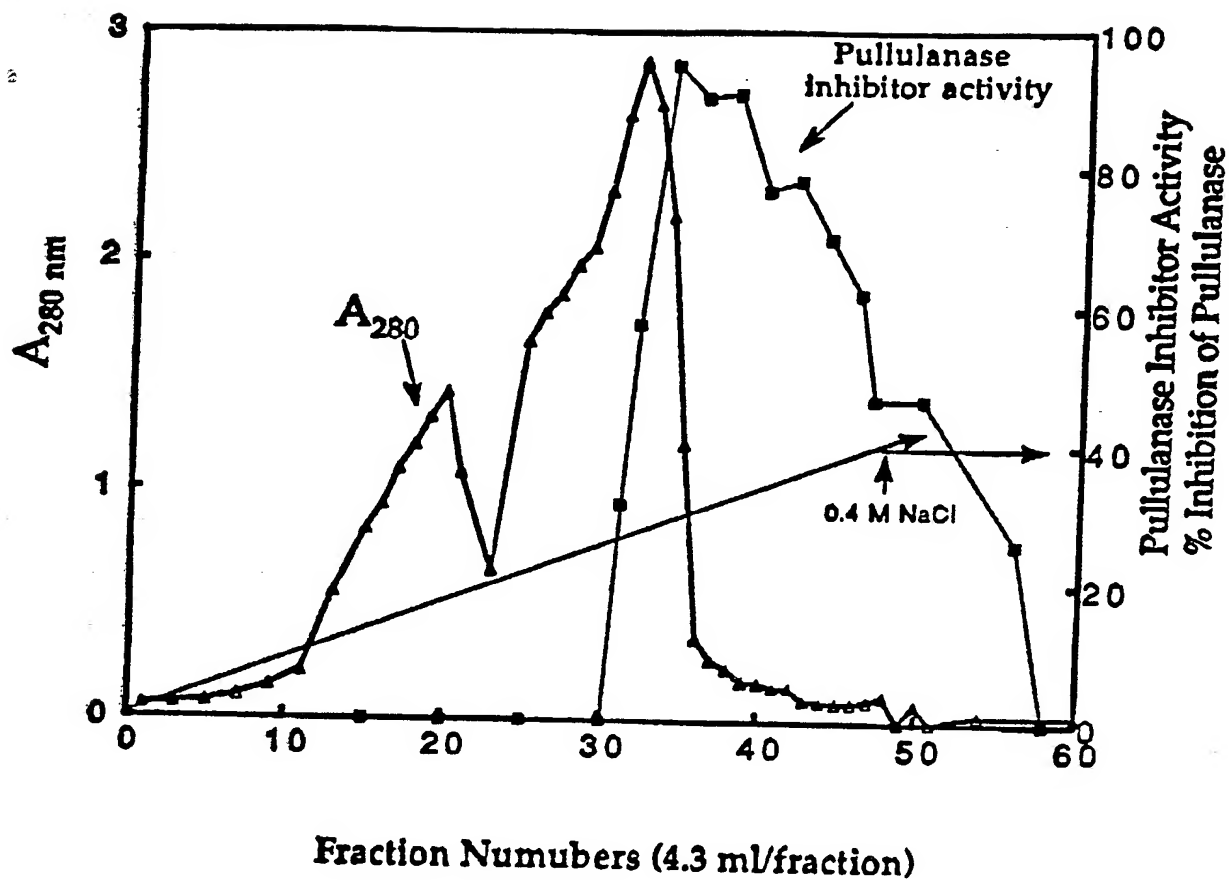


Fig. 42

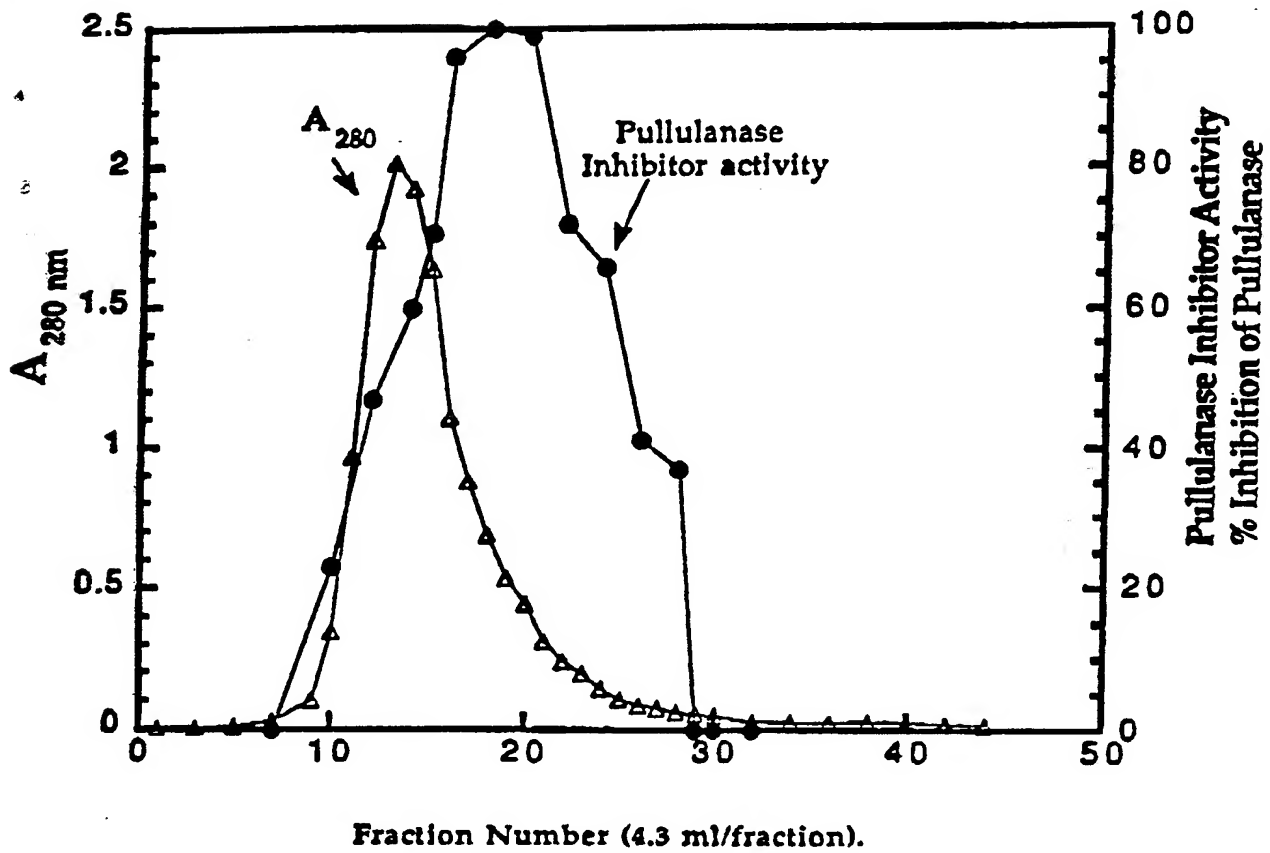


Fig 43

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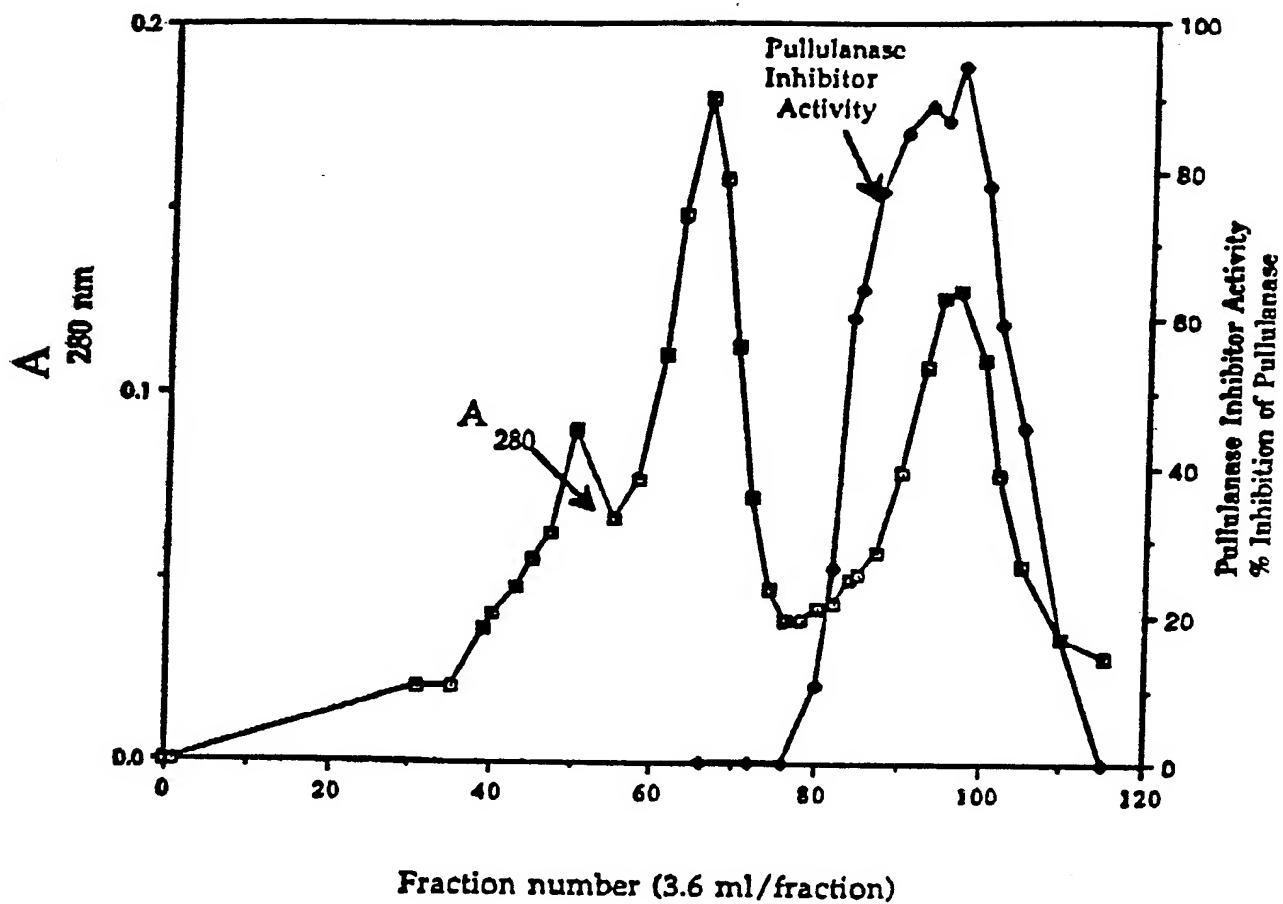


Fig. 44

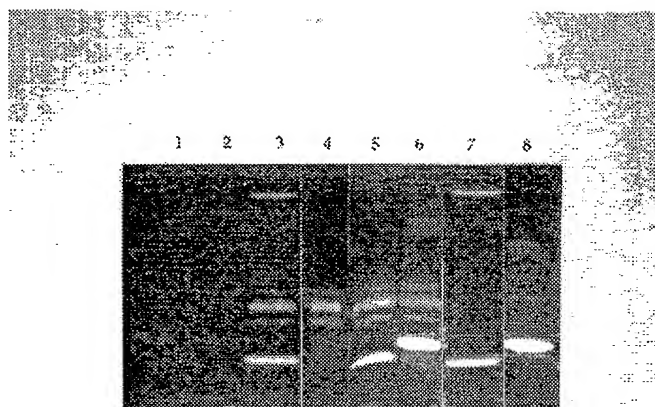


Figure 20.A: Bee venom from *Apis mellifera*

1. Control
2. GSH/NADPH/Glutathione reductase
3. NTS
4. DTT
5. DTT plus *E. coli* thioredoxin
6. DTT plus human thioredoxin
7. NTS, no venom
8. DTT plus human thioredoxin, no venom

Fig. 45

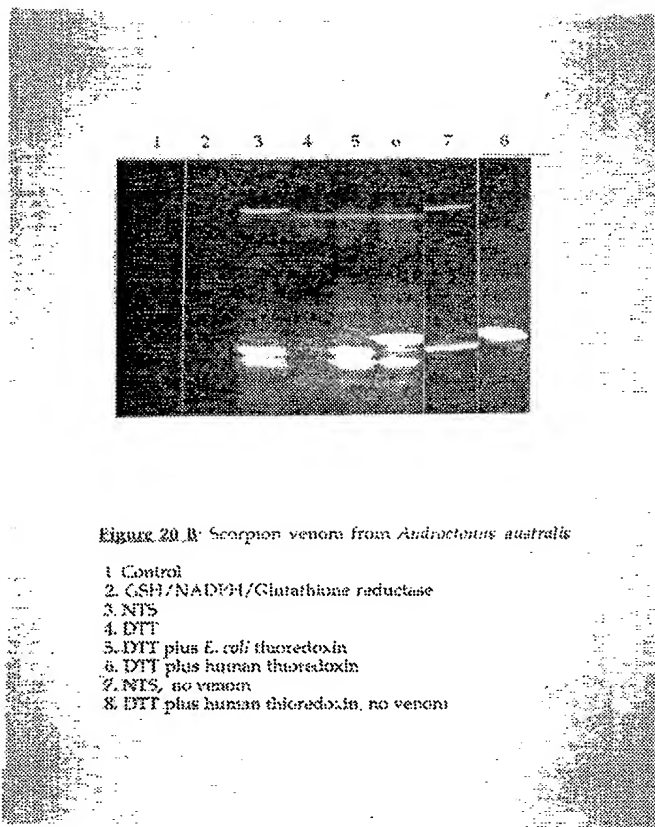


FIG. 46

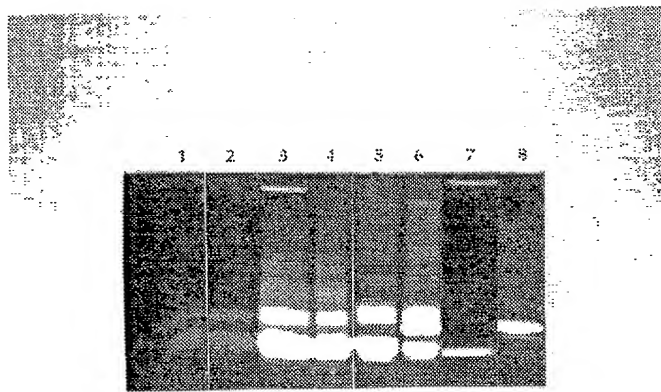


Figure 20 C: Snake venom from *Bungarus multiauctus*

1. Control
2. GSH/NADPH/Catalase reductase
3. NTS
4. DTT
5. DTT plus *E. coli* thioredoxin
6. DTT plus human thioredoxin
7. NTS, no venom
8. DTT plus human thioredoxin, no venom

Fig. 47

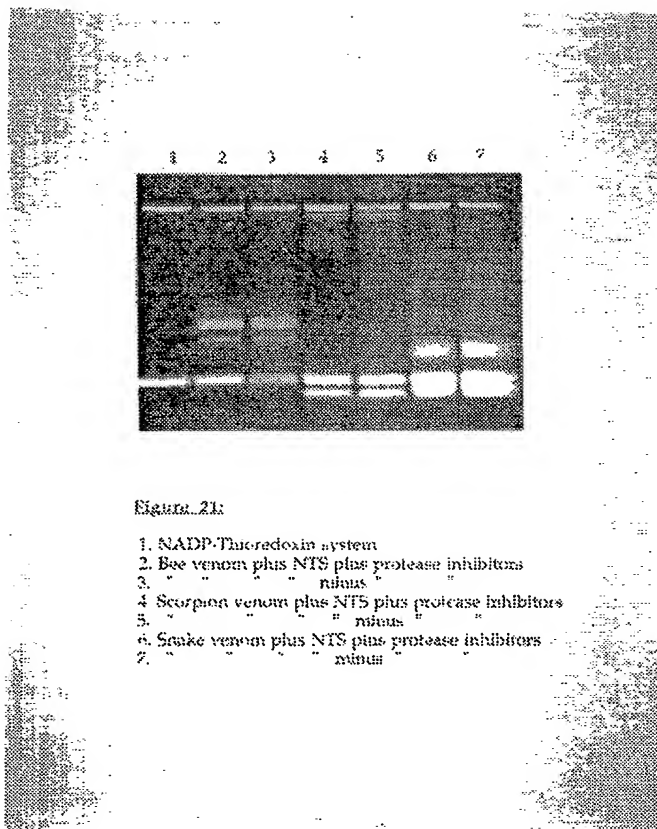


Fig. 48

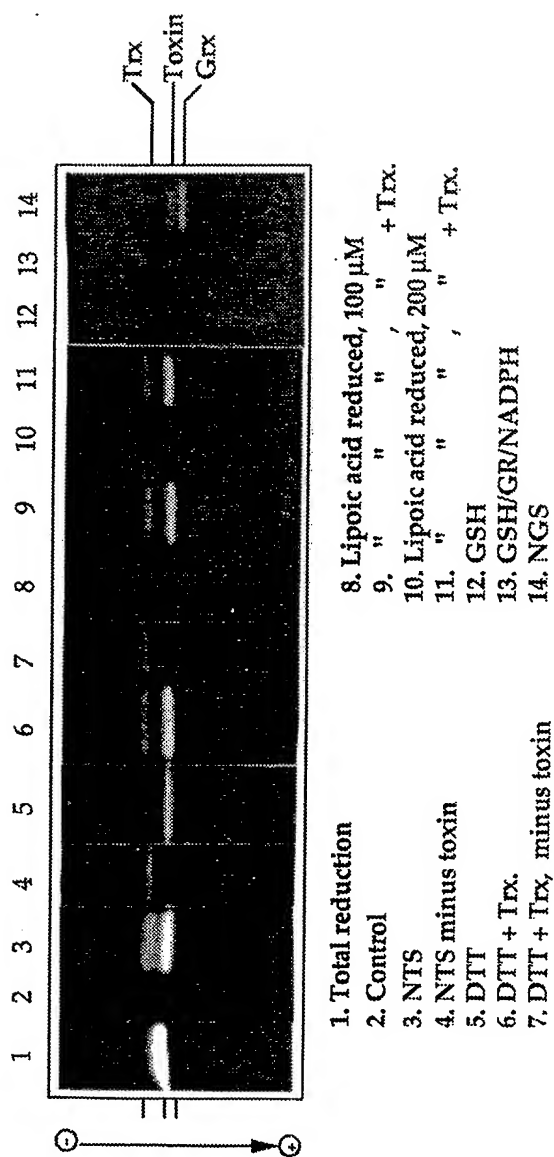
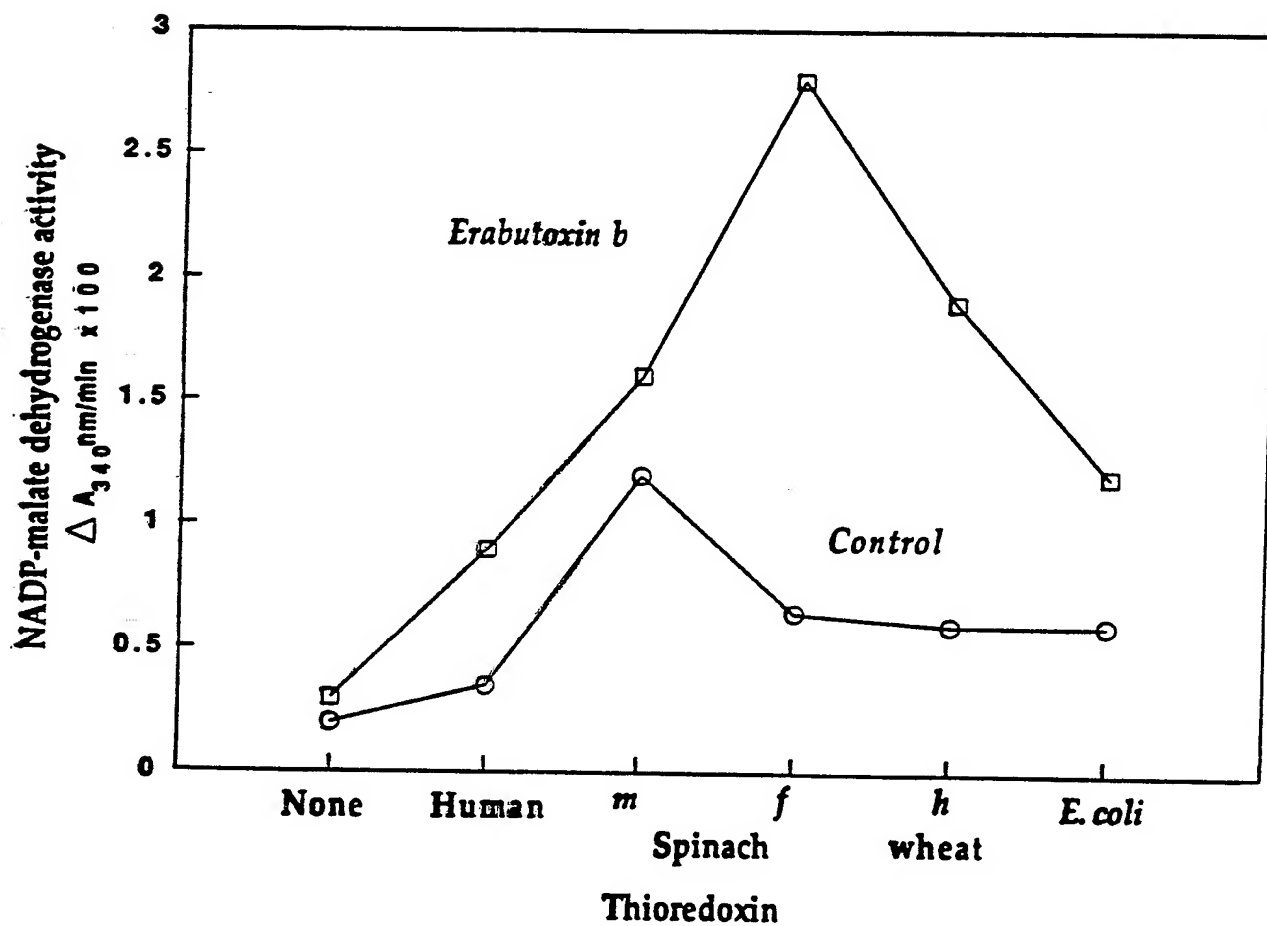


FIG. 49

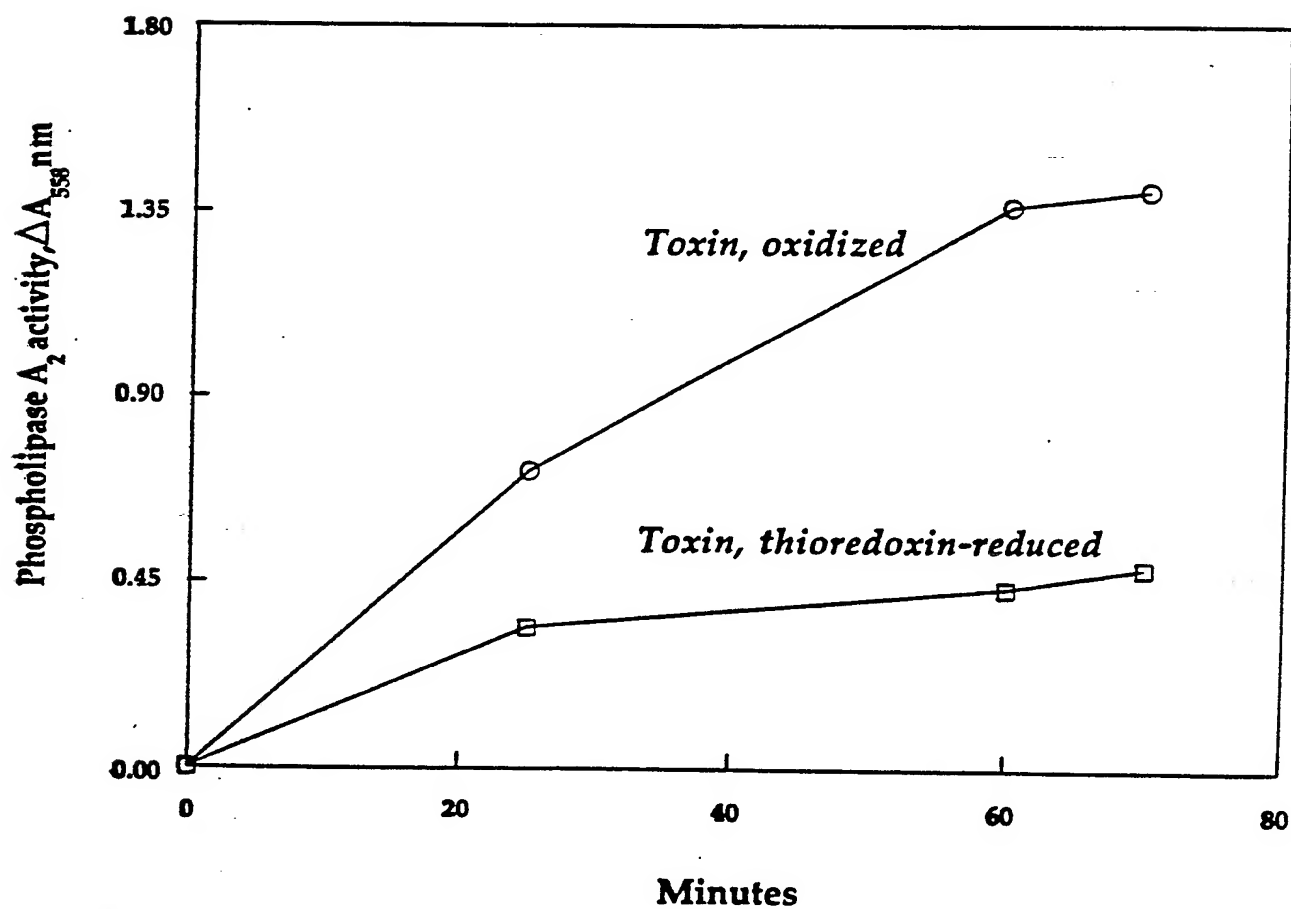
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**Activation of Chloroplast NADP-malate Dehydrogenase  
by Erabutoxin b Reduced with Different Thioredoxin**



**FIG. 50**

**Effect of Thioredoxin-linked Reduction of  
 $\beta$ -Bungarotoxin on its Phospholipase  $A_2$  Activity**



**FIG. 51**

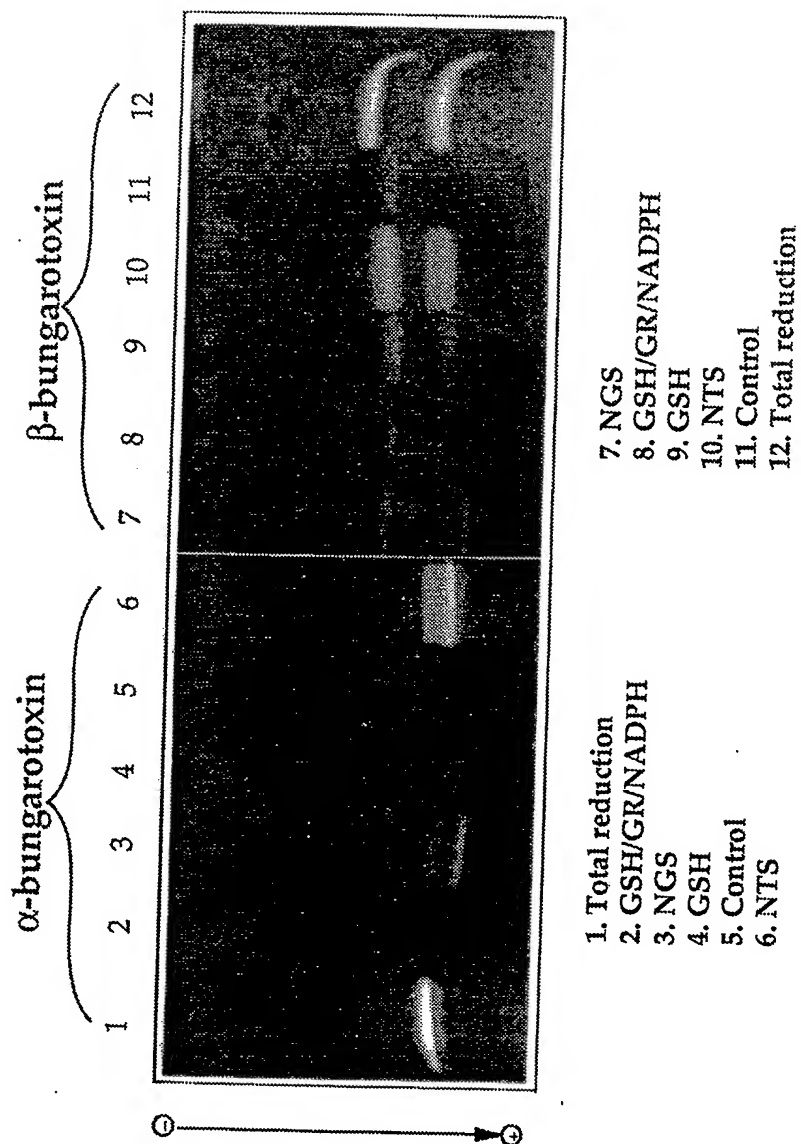


Fig. 52

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US92/08595

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 426/549, 557, 618; 435/18, 69.1, 177, 188, 202, 210, 255; 530/350, 374

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

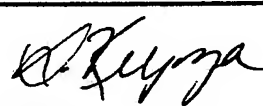
Please See Extra Sheet.

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category* | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No. |
|-----------|---|-----------------------|
| Y         | ARCH. BIOCHEM. BIOPHYS., Volume, 266, No. 2, issued 01 November 1988, Florencio et al., "An NADP/Thioredoxin System in Leaves: Purification and Characterization of NADP-Thioredoxin Reductase and Thioredoxin h from Spinach," pages 496-507, see entire document. | 1-137                 |
| A         | ARCH. BIOCHEM. BIOPHYS., Volume, 271, No. 1, issued 15 May 1989, Crawford et al., "Evidence for Function of the Ferredoxin/Thioredoxin System in the Reductive Activation of Target Enzymes of Isolated Intact Chloroplasts," pages 223-239, see entire document.   | 1-137                 |
| Y         | BIOCHEMISTRY, Volume, 16, No. 1, issued 1977, Howard et al., "Relationship between the Neurotoxicity and Phospholipase A Activity of beta-Bungarotoxin," pages 122-125, see entire document.  | 85-127,136            |

☒ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

|   |     |  |
|---|-----|--|
| * Special categories of cited documents:  | "T" | later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention  |
| "A" document defining the general state of the art which is not considered to be part of particular relevance   | "X" | document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone   |
| "E" earlier document published on or after the international filing date  | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
| "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) | "&" | document member of the same patent family  |
| "O" document referring to an oral disclosure, use, exhibition or other means  |     |  |
| "P" document published prior to the international filing date but later than the priority date claimed  |     |  |

|   |   |
|---|---|
| Date of the actual completion of the international search<br>08 February 1993   | Date of mailing of the international search report<br>26 FEB 1993   |
| Name and mailing address of the ISA/<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. NOT APPLICABLE | Authorized officer<br>ROBERT A. WAX <br>Telephone No. (703) 308-0196 |

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US92/08595

| C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT |  |                       |
|---|--|-----------------------|
| Category*   | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
| Y   | BIOCHIM. BIOPHYS. ACTA, Volume 133, issued 21 February 1967, Yang et al., "The Disulfide Bonds of Cobrotoxin and their Relationship to Lethality," pages 346-355, see entire document.   | 85-119                |
| Y   | CARBOHYDRATE RES., Volume 90, issued 1981, Yamada et al., "Inactive debranching-enzyme in rice seeds, and its activation," pages 153-157, see entire document.   | 78-80                 |
| Y   | FEBS LETT., Volume 124, No. 2, issued February 1981, Wada et al., "Purothionin: A Seed Protein with Thioredoxin Activity," pages 237-240, see entire document.   | 1-84                  |
| Y   | INFECT. IMMUN., Volume 58, No. 12, issued December 1990, Schiavo et al., "An Intact Interchain Disulfide Bond is Required for the Neurotoxicity of Tetanus Toxin," pages 4136-4141, see entire document.                       | 85-137                |
| Y   | JOUR. BIOL. CHEM., Volume 263, No. 18, issued 25 June 1988, Russel et al., "Sequence of Thioredoxin Reductase from Escherichia coli," pages 9015-9019, see entire document.  | 1-137                 |
| Y   | JOUR. BIOL. CHEM., Volume 266, No. 24, issued 25 August 1991, Kobrehel et al., "Role of the NADP/Thioredoxin System in the Reduction of alpha-Amylase and Trypsin Inhibitor Proteins," pages 16135-16140, see entire document. | 1-23                  |
| Y   | JOUR. BIOL. CHEM., Volume 266, No. 14, issued 15 May 1991, Muller et al., "Thioredoxin Deficiency in Yeast Prolongs S Phase and Shortens the G1 Interval of the Cell Cycle," pages 9194-9202, see entire document.             | 24-65,78-84           |
| Y   | JOUR. BIOL. CHEM., Volume 264, No. 24, issued 25 August 1989, Holmgren, "Thioredoxin and Glutaredoxin Systems," pages 13963-13966, see entire document.  | 1-36                  |
| Y   | SCIENCE, Volume 218, issued 01 October 1982, Collins et al., "Colony Defense by Africanized and European Honey Bees," pages 72-74, see entire document.  | 120-127, 136          |
| Y   | NATURE, Volume 337, issued 02 February 1989, Schumacher et al., "Lethality of 'killer' bee stings," page 413, see entire document.   | 120-127,136           |
| Y   | NATURE, Volume 317, issued 19 September 1985, Edman et al., "Sequence of protein disulphide isomerase and implications of its relationship to thioredoxin," pages 267-270, see entire document.                                | 66-76                 |
| Y   | PLANT PHYSIOL., Volume 85, issued 1987, Johnson et al., "Reduction of Purothionin by the Wheat Seed Thioredoxin System," pages 446-451, see entire document.   | 1-84                  |
| Y   | METHOD ENZ., Volume 45, issued 1976, Birk, "Proteinase Inhibitors from Plant Sources," pages 695-739, see entire document.   | 14-23                 |

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/US92/08595

## A. CLASSIFICATION OF SUBJECT MATTER:

IPC (5):

C12N 9/28, 9/96, 11/02; C12Q 1/34; A21D 10/00; A21D 13/00; A23L 1/18, 1/168, 1/172; A61K 35/7, 35/80; C07K 3/00, 13/00, 15/00, 17/00

## A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

426/549, 557, 618; 435/18, 69.1, 177, 188, 202, 210, 255; 530/350, 374

## B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, STN(Biosis)

search terms: thioredoxin, thiol redox, thioredoxin h, thioredoxin reductase, glutaredoxin, glutathione, amylase, reduc?, lipoic acid, subtilisin, inhibit?, glutenin, dough, semolina, wheat, rye, gliadin, bowman-birk, soybean, trypsin inhibitor, triticale, albumin, 2s, pullulanase, yeast, trx, snake venom, snake neurotoxin, facilitatory neurotoxin, bungarotoxin, phospholipase A2, erabutoxin, bee, scorpion, venom, toxin, rice, sorghum, corn

## BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

I. Claims 1-5, 14-23, 24-30, 31-37, drawn to a method and composition of reducing proteins, using a reductase or a protein reaction product, classified in Class 435, subclass 188+.

II. Claims 6-12, and 13, drawn to a composition and method of reducing an amylase inhibitor protein having disulfide bonds, classified in Class 435, subclass 177 or 202.

III. Claims 38-41, 42, 43-49, 53 and 62-65, drawn to a method of inactivating an enzyme inhibitor protein in a food product, classified in Class 426, subclass 18, 549 and 618.

IV. Claims 50, 51 and 52, drawn to a method for producing an improved gluten, classified in Class 530, subclass 374.

V. Claims 54-56, 57-59 and 60-61, drawn to a yeast cell, classified in Class 435, subclass 255.

VI. Claims 66-77, drawn to a method of reducing disulfide bonds, classified in Class 350, subclass 69.

VII. Claims 78-79 and 80, drawn to a method of increasing the activity of the enzyme pullulanase, classified in Class 435, subclass 210.

VIII. Claims 81-84, drawn to a method of improving the characteristics of a cooked pasta, classified in Class 426, subclass 557.

IX. Claims 85-119, drawn to a method of reducing a snake neurotoxin protein, classified in Class 530, subclass 350.

X. Claims 120-128 and 136, drawn to a method of reducing a bee venom toxic protein, classified in Class 530, subclass 350+.

XI. Claims 129-135 and 137, drawn to a method of reducing a scorpion venom toxic protein, classified in Class 530, subclass 350+.

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US92/08595

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
  
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
  
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:  
Please See Extra Sheet.

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
  
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐

The additional search fees were accompanied by the applicant's protest.

☒

No protest accompanied the payment of additional search fees.